A clinical and serological study of adult and juvenile idiopathic inflammatory myopathy

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A CLINICAL AND SEROLOGICAL STUDY OF ADULT AND JUVENILE IDIOPATHIC INFLAMMATORY MYOPATHY

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For my mother

GLOSSARY

List of abbreviations

A. Methods

AMP	Ammonium persulphate
BS3	Bis(sulphosuccinimidyl)suberate
dFCS	dialysed Foetal Calf Serum
DMP	Dimethyl pimelimidate-2 HCL
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoabsorbant assay
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
HCL	Hydrogen chloride
HEp-2	Human epithelial cell line
IB	Immunoblotting
IIF	Indirect immunofluorescence
IPP	Immunoprecipitation
K562	Human chronic myelogenous leukaemic cell line
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MBq	Megabecquerel
NaCl	Sodium Chloride
$NaHCO_3$	Sodium Bicarbonate
PBS	Phosphate-buffered Saline
RPMI	Rosewell Park Memorial Institute 1640 medium
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered Saline
TEMED	N,N,N^1,N^1-tetramethylethylenediamine
Tris	Tris[hydroxymethyl]aminomethane
WB	Western Blotting

GLOSSARY

List of abbreviations

B. Clinical

AIP	Acute interstitial pneumonia
ANA	Anti-nuclear autoantibody
ANoA	Anti-nucleolar autoantibody
AOMIC	Adult Onset Immunogenetic Collaboration
ARS	Aminoacyl-tRNA
ASS	Anti-synthetase syndrome synthetases
ATP	Adenosine-5'-triphosphate
ATS	American Thoracic Society
BIRD	Bath Institute for Rheumatic Diseases
CADM	Clinically-amyopathic dermatomyositis
CAT	Cutaneous Assessment Tool
CHAQ	Childhood Health Assessment Questionnaire
CK	Creatinine kinase
CMAS	Childhood Myositis Assessment Scale
COP	Organising pneumonia
CRP	C-reactive protein
CTD	Connective Tissue Disease
DM	Dermatomyositis
DNA	Deoxyribonucleic acid
DPLD	Diffuse parenchymal lung disease
EMG	Electromyogram
ENA	Extractable nuclear antigen
ER	Endoplasmic reticulum
ERS	European Respiratory Society
ESR	Erythrocyte sedimentation rate
ET-1	Endothelin-1
GI	Gastrointestinal
HLA	Human leucocyte antigen
HRCT	High-resolution computerised tomography
IBM	Inclusion body myositis
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
IIM	Idiopathic inflammatory myopathy

GLOSSARY

List of abbreviations

B. Clinical

JDM	Juvenile dermatomyositis
JDRR	Juvenile dermatomyositis Registry and Repository
LDH	Lactate dehydrogenase
MAA	Myositis-associated autoantibody
MCTD	Mixed connective tissue disease
MDA5	Melanoma-differentiation associated gene 5
MH	Mechanic's hands
MHC	Major histocompatibility complex
mRNA	messenger ribonucleoprotein
MSA	Myositis-specific autoantibody
NSIP	Non-specific interstitial pneumonia
NURD	Nucleosome remodelling deacetylase
NXP-2	Nuclear matrix protein-2
PBC	Primary biliary cirrhosis
pDCs	Plasmacytoid dendritic cells
PGA	Physician's global assessment
PM	Polymyositis
RANGAP	Ran GTPase activating protein
RNHRD	Royal National Hospital for Rheumatic Diseases
RP	Raynaud's phenomenon
SAE	Small ubiquitin like modifier enzyme
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SRP	Signal recognition particle
SSc	Systemic sclerosis
SUMO	Small ubiquitin-like modifiers
TIF1-γ	Transcriptional intermediary factor 1-gamma
TNF	Tumour necrosis factor
tRNA	transfer ribonucleoprotein
UV	Ultra-violet
VAS	Visual analogue score
VCAM-1	Vascular cellular adhesion molecule-1
UIP	Usual interstitial pneumonia

ABSTRACT

Introduction: The idiopathic inflammatory myopathies (IIM): dermatomyositis (DM) and polymyositis (PM) have been historically defined by broad clinical and pathological criteria. These conditions affect both adults and children with clinical features including muscle weakness, skin disease and internal organ involvement. Using a clinico-serological approach DM and PM can be defined into more homogeneous subsets. Myositis-specific autoantibodies (MSAs) are directed against cytoplasmic or nuclear components involved in key regulatory intra-cellular processes including protein synthesis, translocation and gene transcription. Over the last few years MSAs have been better characterised including autoantibodies directed against the aminoacyl tRNA-synthetase (ARS) enzymes, the signal recognition particle and the Mi-2 protein.

Aim: The overall aim of this thesis is to describe a comprehensive clinical and serological study of adult and juvenile IIM. Autoantigen targets including novel specificities were identified using protein immunoprecipitation.

Results: The first part of this thesis is a descriptive study on known myositis autoantibodies in adult IIM, confirming the significant association of interstitial pneumonia with anti-ARS, severe myopathy with anti-SRP, and classic DM with anti-Mi-2 serotype. In the next section, new autoantigen systems in adult IIM are described including a new anti-ARS (anti-Zo) in the anti-synthetase syndrome. Further autoantibodies directed against small ubiquitin-like modifier enzyme and a p155/140 autoantigen are major serological subsets in adult DM, the latter significantly associated with malignancy. The final section outlines a large serological study of juvenile DM (JDM) and JDM-overlap showing the frequency and clinical associations of MSAs and myositis-associated autoantibodies, including work on two new major subsets anti-p155/140 and anti-p140, which appear to define more severe disease.

Conclusion: The work in this thesis highlights the importance of autoimmunity in IIM and suggests a new approach where MSAs can classify patients into clinical syndromes, which predict outcomes and may as a result influence treatment strategies.

CHAPTER 1
INTRODUCTION

1.1 Background

The idiopathic inflammatory myopathies (IIM) are a group of rare autoimmune diseases that affect both adults and children. The major subtypes of IIM are polymyositis (PM) and dermatomyositis (DM) that are heterogeneous conditions characterised by skeletal muscle weakness, biochemical or histological evidence of muscle inflammation, skin lesions and systemic organ involvement (1, 2). Morbidity outcomes relate to well-known complications, e.g. cancer-associated myositis and interstitial pneumonia. In children there is also a significant morbidity associated with vasculopathic organ involvement, soft tissue calcinosis and skin ulceration. Response to immunosuppressive therapies is variable and patients may die from disease or treatment complications, or remain very disabled. The associations with cancer and interstitial pneumonia make IIM an important autoimmune model to investigate further. The precise aetiology is unknown, mechanisms require further elucidation but the interaction between immunogenetic and environmental factors appears to play a major role (3). Like other systemic autoimmune diseases including systemic lupus erythematosus (SLE) and systemic sclerosis (SSc), IIM is characterised by the production of autoantibodies. The identification and characterisation of autoantibodies is an important cornerstone in the diagnosis of autoimmune connective tissue disease (CTD) (4, 5). Autoantibodies and corresponding target autoantigens are the subject of considerable interest, and there is increasing evidence to support a critical association between genotype, serotype and clinical phenotype in both adult and juvenile myositis (6-10).

1.2 Classification and diagnosis

In 1975, *Bohan and Peter* first proposed criteria to aid the diagnosis and classification of PM and DM (1, 2). The five adapted criteria proposed were as follows:

1. Symmetrical proximal weakness of the limb or pelvic girdle and anterior neck flexors progressing over weeks to months, with or without dysphagia or respiratory muscle involvement.
2. Elevation of muscle enzymes, including creatinine kinase (CK).
3. Abnormal electromyogram (EMG) with a triad of
    - Short duration, small amplitude polyphasic motor unit potentials.

- Fibrillations, positive sharp waves, increased insertional irritability.
- Spontaneous, bizarre, high frequency repetitive discharges.

4. Abnormal muscle biopsy demonstrating inflammatory cell infiltrate, muscle degeneration, regeneration or necrosis.
5. Typical cutaneous signs of DM including
   - Heliotrope rash – violaceous erythema on upper eyelids
   - Gottron's papules – violaceous keratotic papules on extensor aspects of fingers or elbows or knees.

Exclusion criteria: absence of other forms of myopathy e.g. inclusion body myositis, metabolic, inherited or infectious forms of myopathy.

Bohan and Peter suggested five subsets of myositis: PM, DM, cancer-associated myositis, juvenile myositis and myositis overlapping with CTD. *Bohan and Peter* also described that patients can develop cutaneous features of DM, which may precede the onset of myopathy (2).

- Definite DM requires all five criteria.
- Definite PM requires first four criteria (without rash).

- Probable disease requires three criteria out of first 4 criteria (for DM, one of which is rash i.e. criteria 5).

- Possible disease requires two criteria out of first 4 criteria (for DM, criteria 5 plus one other.

Overlap myositis: must fulfil criteria for their main CTD e.g. systemic sclerosis (11) or systemic lupus erythematosus (12), at least two of criteria 1 – 4, and at least one myositis-specific or associated autoantibody (see section 1.6.4).

Tanimoto *et al* published further classification criteria for PM / DM in 1995 (13).

1. Skin lesions:
   - Heliotrope rash.
   - Gottron's papules (extensor aspect of fingers, elbows or knees.
2. Systemic inflammatory signs (elevated erythrocyte sedimentation rate, C-reactive protein, pyrexias, weight loss).
3. Non-erosive arthritis.

4. Myositis-specific autoantibodies e.g. anti-JO-1
5. Elevated skeletal muscle enzymes.
6. Myalgia at rest or with contraction.
7. Proximal muscle weakness.
8. Myopathic changes on EMG.
9. Myositis changes on muscle biopsy.

- For a diagnosis of definite PM – at least four from item list 2 – 9.
- For a diagnosis of definite DM – at least four from item list 2 – 9 plus at least one feature from item 1.

The IIM spectrum also includes a further subset, sporadic inclusion body myositis (IBM) and *Griggs et al* have subsequently proposed classification criteria (14). Unlike other IIM, sporadic IBM causes slowly progressive myopathy with atrophy with a distinctive pattern of muscle involvement that may be asymmetrical and affect distal rather than proximal muscle groups, and is often unresponsive to conventional immunomodulatory therapy (15). Historically, IBM cases have been seen and managed by Neurologists. Clinicians now recognise histopathology and other more specific investigations are required to exclude IBM, hereditary and other non-immune mediated myopathies when applying the *Bohan and Peter* criteria. This study has not investigated this subgroup of patients and in this thesis IIM refers purely to PM and DM cases.

1.3 Epidemiology

The true frequency of PM and DM as stand-alone disorders or in association with other CTD is unknown. Reports of incidence and prevalence are limited based on different study populations. Estimates based on the diagnostic criteria proposed by *Bohan and Peter* (1, 2), may not be accurate due to difficulties distinguishing PM and IBM. Reports show an annual incidence of 2-7 / million population (16-18). The mean age of onset is 50 years affecting women more than men. Juvenile dermatomyositis (JDM) is the most common IIM of children and the reported incidence is 0.8-4.1 per million children per year (18-20). In contrast to adults, juvenile PM is rare (21), whereas JDM overlap with other connective tissue disease in particular SSc and SLE is more common.

1.4 Clinical features

IIM is characterised by a spectrum of clinical features including skeletal muscle weakness, skin lesions and systemic organ involvement, in particular interstitial pneumonia and a risk of cancer in adults. Children with JDM share some clinical features with adult patients in terms of muscle disease and characteristic skin lesions. Certain clinical features including skin ulceration, calcinosis and gastro-intestinal involvement are more common in children and have been proposed as predictors of severe disease course in JDM (21-25). In contrast to adults with DM, both interstitial pneumonia and cancer-associated myositis are very rare in JDM. Delays in diagnosis, in particular recognising active muscle or lung disease and poor response to treatment mean that patients are often left disabled from irreversible damage.

1.4.1 Myositis

IIM can present with progressive symmetrical muscle weakness that usually develops over a few weeks to months. The proximal muscle groups such as the shoulder girdle, pelvic girdle, thigh muscles and neck flexors are typically involved. In severe cases, other muscle groups can be involved including chest wall weakness, which can compromise respiratory function. At onset, patients rarely complain of muscle pain but experience difficulties with activities of daily living, for example, rising from a chair, walking up the stairs and raising their arms. Depending on the severity of the case and the response to treatment muscle atrophy can develop that leads to severe irreversible weakness.

1.4.2 Cutaneous manifestations

Classic DM skin lesions are generally present at the time of diagnosis and in some cases may precede the development of muscle inflammation or other organ involvement (see Section 1.5.3) (26). Gottron's sign is an erythematous, scaly plaque-like lesion usually found over the dorsal surface of the metacarpophalageal or proximal interphalangeal joints and the extensor surfaces of the knees or elbows. Heliotrope rash is a purple violaceous eruption of the upper eyelids, often with cutaneous oedema. More extensive skin involvement includes the V-sign and Shawl-sign rash over the anterior triangle of the neck, and the back of the neck and shoulders respectively. Erythroderma can develop on the face (malar distribution or forehead). All these rashes are diffuse, flat and erythematosus and may be photosensitive. The skin lesions can have a poikilodermatous appearance with a combination of atrophy, pigment changes and telangiectasia. Periungal erythema

with abnormal dilated nailfold capillaries is another feature. Mechanic's hands are a characteristic cutaneous manifestation that is pathonomonic of the clinical and serological subset termed the anti-synthetase syndrome (see Section 1.5.1). The description refers to dry, cracked or fissured skin with hyperkeratosis over the lateral aspects of the inter-digits especially the index and middle fingers. Certain features secondary to persistent cutaneous vasculitis are more common in JDM, in particular generalised oedema, skin ulceration and soft tissue calcification (calcinosis) (21-25).

1.4.3 Interstitial pneumonia

Diffuse parenchymal lung disease (DPLD) is a major cause of morbidity and mortality in IIM (27). Primary lung involvement or ventilatory failure secondary to muscle weakness is common, more so in adult IIM (28-30). Interstitial pneumonia is the most frequent sub-type of DPLD in IIM, with studies showing an overall frequency of between 20-60% (29, 31). Interstitial pneumonia (also termed interstitial lung disease) are a heterogeneous group of non-malignant and non-infectious lung disorders that comprise of a number of clinico-pathological entities that are sufficiently different to be designated as separate diseases. The characteristic hallmark is that of varying patterns of inflammation and fibrosis affecting the lung parenchyma.

In 2002 the American Thoracic Society and the European Respiratory Society (ATS/ERS) published the consensus classification for interstitial pneumonia that is equally applicable in CTD (32). Based on radiological and histopathological patterns, interstitial pneumonia can be further subdivided into several subsets including usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP), organising pneumonia (OP) and acute interstitial pneumonia (AIP), which are recognised in CTD (32, 33). The histological hallmark of UIP is the presence of fibroblastic foci with a subpleural, basal and peripheral distribution (32, 34). The most important differential pattern of UIP, particularly in CTD is NSIP, which has a similar subpleural and basal distribution. *Katzenstein et al* first described NSIP characterised by cellular and fibrotic changes with the absence of dense fibrosis or fibroblastic foci (35). With the advent of high-resolution computerised tomography (HRCT), interstitial pneumonia patterns in CTD can be categorised radiologically with good histological correlation (36, 37).

NSIP has been reported to be the most frequent subtype on both HRCT and lung biopsy in IIM (38). AIP, COP and UIP have also been reported and identified as

patterns responsible for acute or rapidly progressive interstitial pneumonia with AIP and UIP associated with the worst prognosis (28-31, 33, 39). It is well recognised that interstitial lung involvement may be the first or predominant organ manifestation preceding muscle or skin disease, particularly in the anti-synthetase syndrome (see Section 1.5.1) and in patients with clinically-amyopathic DM (CADM) (see Section 1.5.3) (29, 31, 40-42).

1.4.4 Arthritis and overlap features

Non-erosive inflammatory arthritis is a frequent manifestation of IIM, particularly in anti-synthetase syndrome and overlap conditions such as SSc, mixed connective tissue disease and SLE. Raynaud's phenomenon characterised by vasospasm and structural changes of the digital microvasculature is a common feature. Joint disease with contractures and lipoatrophy of soft tissues is more frequent in JDM particularly in patients with overlap scleroderma. General features include fatigue, weight loss and fever.

1.4.5 Other organ manifestations

Other organs can be involved including the striated muscles of oropharyngeal and upper oesophagus causing dysphagia, and gastro-intestinal tract vasculopathy leading to ischaemic ulceration with pain and bleeding, the latter a more common complication in JDM (43). Cardiac involvement including myocarditis has been reported with subsequent conduction defects and ventricular failure (44, 45). Although rare, cardiac disease confers a high risk for mortality in patients with IIM (27, 44).

1.5 Specific clinical subsets

1.5.1 Anti-synthetase syndrome

The anti-aminoacyl tRNA synthetase (anti-ARS) autoantibodies (see Section 1.6.2) define the anti-synthetase syndrome recognised as a spectrum of myositis, interstitial pneumonia, non-erosive arthritis, fever, Raynaud's phenomenon and mechanic's hands (46). Patients may have different clinical features within the syndrome, but overall there is a significant association with interstitial pneumonia and studies have described the frequency to be as high as 95% (28-30, 47, 48).

1.5.2 Cancer-associated myositis

The association between cancer and adult DM is well recognised particularly in the older age groups (49-54). Because IIM can have a chance association with malignancy, cancer-associated myositis is defined by a temporal association of less than three years between cancer and DM onset (50, 51). The association with PM is uncertain with some studies reporting an increased relative risk but some not (53, 55, 56). Hill *et al* conducted a pooled analysis of published national data from Sweden, Denmark, and Finland on all patients with DM and PM (over the age of 15 years). The study calculated standardised incidence ratios (SIR) for individual cancer sites for dermatomyositis and polymyositis separately, using national cancer rates by country, sex, age, and date. The frequency of cancer in the DM cohort was 32% (58% of which developed cancer after the diagnosis of DM), with a SIR of 3.0. The most common cancer observed was ovarian followed by lung, pancreatic, non-Hodgkin lymphoma stomach and colorectal. 15% of PM cases were associated with cancer (70% developed after the diagnosis of PM), with a SIR of 1.3. PM was associated with a higher risk of non-Hodgkin lymphoma, then lung and bladder malignancy. In both DM and PM, the risk of malignancy was highest at the time of myositis diagnosis (within the first year) with a markedly reduced relative risk over time. For DM the SIR of cancer by year after diagnosis of myositis was 13.5 (0-1 year), 2.5 (2-5 years) and 1.4 (>5 years). In the PM group the SIR of cancer by year after diagnosis of myositis was 2.6 (0-1 year), 1.5 (2-5 years) and 0.9 (>5 years). In the DM cohort the number of cancers recorded in patients under the age of 44 years was small, and there was no increase in risk in PM patients (53). In contrast to adults, JDM appears not to be associated with malignancy (53, 57). Historically, cancer-associated myositis has not been associated with specific myositis autoantibodies, but recent reports including work described in this thesis have described a novel autoantibody marker in this clinical subset *(*see section 1.6.2).

1.5.3 Clinically-amyopathic dermatomyositis (CADM)

Patients may only develop DM skin changes with no clinical evidence of myopathy, termed DM sine myositis or amyopathic DM (ADM) (40). Clinically-amyopathic DM (CADM) is a designation for patients who either have ADM or those patients who have DM skin lesions, no muscle weakness but evidence of sub-clinical myopathy on further testing (muscle enzymes, EMG, imaging or biopsy), termed hypomyopathic DM.

1.6 Treatment

The treatment of both PM and DM is often empirical and in general is influenced by clinical experience rather than robust clinical data. There remains a paucity of standardised clinical trials due to the relative rarity of PM and DM. In addition, perhaps due to historical classification tools including the *Bohan and Peter* criteria, IIM is a heterogeneous condition, which makes designing trials and interpreting treatment outcomes in different disease subtypes difficult. For example, optimal treatment strategies for ASS with lung disease may be different to other subgroups, CADM or immune-mediated necrotising myopathy. Moreover, the absence of standardised disease activity and outcome measures has made the assessment of treatment response difficult. Classifying patients into more homogenous subsets based on serotype / clinical phenotype, and the advent of validated myositis disease activity tools (see section 1.7) will facilitate the design of clinical trials, which can capture clinically significant changes.

Based on personal clinical experience and limited trial data there are several treatments that are effective treating patients with IIM.

Glucocorticoids

Prednisolone is recognised as the first line treatment for PM or DM including those with internal organ complications e.g. ASS with interstitial lung involvement. This is primarily based on clinical experience rather than trial data. In the majority of cases, the initial dose in 0.5-1mg/kg daily, and depending on clinical response the dose is weaned over a number of months. Patients may remain on low dose prednisolone for a number of years, particularly those with ASS and ILD, which appears to be very steroid responsive but also steroid dependant with disease flares on steroid withdrawal (48) (personal observation). In severe cases of myositis with internal organ involvement (including those with gastrointestinal involvement e.g. vasculopathy or pharyngeal dysfunction), patients may require induction with intravenous methylprednisolone pulses.

Specific immunomodulatory therapy

In patients who respond to steroids, the goal is to induce disease control and thus remission as quickly as possible at the same as minimising steroid requirements. In mild to moderate cases, for example those with purely muscle and / or skin disease, this may be achieved with either Azathioprine or Methotrexate (58-61). An alternative to Azathioprine and Methotrexate is Ciclosporin, the latter two agents

have also been compared in a small randomised controlled trial, with no significant difference observed, although appeared effective (62). However, the side effect of profile may be higher with Ciclosporin, which often limits its use in clinical practice. In moderate to severe cases, induction therapy in combination with pulsed / oral prednisolone may be required. The efficacy of intravenous immunoglobulin (IV IG) has been demonstrated, especially in DM, in a randomised controlled trial (63). In DM patients who response to standard regimes with prednisolone and Azathioprine / Methotrexate is inadequate or in those patients with severe disease at presentation (not complicated by interstitial pneumonia), IV IG is the preferred agent of choice. Repeated infusions may be required to achieve disease control followed by maintenance therapy as outlined above. In severe cases, including those with significant interstitial pneumonia and / or severe necrotising myopathy, based on personal clinical experience, IV Cyclophosphamide (CYC) may be a useful adjuvant therapy. In general, induction with 4-8 pulsed infusions given every 3-4 weeks is an optimal strategy. If this treatment fails or is contra-indicated, the novel biologic agent Rituximab (monoclonal antibody against CD20-positive B cells) appears to be an attractive treatment option, especially in myositis-autoantibody-positive refractory patients e.g. ASS patients with interstitial pneumonia and DM (64). A multi-centre / international randomised placebo controlled has recently completed recruitment (*http://www.edc.gsph.pitt.edu/rimstudy/index.html*). There is now preliminary data for newer agents in moderate disease or as maintenance therapy following induction with IV IG, IV CYC or Rituximab. Mycophenolate Mofetil inhibits the *de novo* pathway of nucleotide synthesis leading to an anti-proliferative affect directed against T and B lymphocytes. It appears to be a useful agent particularly in those patients where Azathioprine or Methotrexate is ineffective or poorly tolerated. There is also preliminary data that suggests Mycophenolate may be beneficial in refractory IIM cases associated with interstitial pneumonia (65-67). A further option is Tacrolimus, a calcineurin inhibitor that blocks T cell signal transduction and IL-2 signalling. This drug can be effective in some difficult-to-treat cases of polymyositis / ASS, especially in patients with interstitial lung disease (68, 69).

1.7 Assessment Tools

In a clinical setting, proximal myopathy is often assessed by physician tested medical research council (MRC) scale for muscle strength. Manual muscle testing, however, has a number of limitations. One limitation is that the MRC scale is an ordinal scale with disproportional distances between grades. Another limitation of the MRC scale is that the scoring depends on the judgment of the examiner. Finally,

with the 6-point ordinal MRC scale, it is difficult to identify relatively small but clinically relevant changes in muscle strength. Alternative more objective tests to assess proximal muscle function have been devised, including two isotonic tests (1 kg arm lift for upper limb function and a 30 second chair stand test for lower limb function. These tests exhibit excellent test-retest reliability, demonstrate construct validity and are responsive to changes in disease activity in IIM (70). New tools have been devised to standardise the conduct and reporting on IIM clinical trials. Two tools, known as the myositis intention to treat index (MITAX) and the myositis disease activity assessment visual analogue scale (MYOACT), have been developed to measure activity in patients with IIM (*www.niehs.nih.gov/research/resources/collab/imacs/docs/activity/MDAAT_Scoring_2009.pdf*). In addition, the myositis damage index (MDI) has been devised to assess the extent and severity of damage developing in different organs and systems. These measures have been reviewed by the International Myositis Assessment and Clinical Studies (IMACS) group and have been found to have good face validity (71). A recent study has demonstrated that the myositis disease activity assessment tool (MITAX and MYOACT) has good interrater reliability and validity in clinical practice (72). In addition to these tools, specific disease assessment scales have also been designed in juvenile IIM, which are used in routine clinical practice, as well as research studies (see section 5.1.2 and appendix 5.5).

1.8 Pathogenesis and aetiology

It is becoming increasingly clear that IIM is a useful model to study the relationship between immunogenetic profiles, autoimmune targets and clinical phenotype in CTD. The development of IIM appears to be secondary to the combination of genetic factors and exposure to environmental agents that may initiate specific autoimmune responses in certain individuals.

1.8.1 Pathogenesis

Classic descriptions

Cellular and humoral autoimmune mechanisms that target skeletal muscle, the lungs, the skin, and associated microvasculature are implicated in the pathogenesis of IIM. In broad terms, in DM, immune responses are said to primarily target the microvascular endothelium leading to activation of the complement cascade, upregulation of cytokines and chemokines, and thus endothelial damage. Inflammatory lesions with B and CD4 positive T cells are predominately found in perivascular and perimysial regions with perifascicular atrophy. Endothelial

hyperplasia and fibrin thrombi are seen in both endomysial and dermal capillaries (17, 73, 74). Cytokines are released, which leads to upregulation of vascular-cell adhesion protein-1 (VCAM-1) and intercellular adhesion molecule (ICAM-1), which promotes endothelial damage. Historically in PM, myocytotoxicity is said to be mediated by cellular responses where clonally expanded CD8 positive T cells are primed against muscle fibres expressing MHC class I antigens leading to an inflammatory cell infiltrate that invades muscle fascicles (17, 75). Previous descriptions have suggested B cells are sparse within the inflammatory cell infiltrate of PM specimens (76).

Shared cellular and humoral mechanisms

It is now becoming increasingly clear that both T and B cell responses occur in both subtypes of IIM. Both CD4 and CD8 T cells have been demonstrated in PM and DM muscle tissue (77). In addition, cytokines, chemokines and their receptors, are strongly expressed by endothelial and inflammatory cells in both subtypes. CD4+ cells previously characterised as T cells have been identified as plasmacytoid dendritic cells (pDCs). Large-scale gene expression studies have confirmed a type 1 interferon (IFN) signature with abundant pDCs in DM muscle and skin (78, 79). More recently, type 1 IFN α/β producing pDCs have also identified in PM muscle (80), highlighting the potential role of this cytokine system in the pathogenesis of IIM. Furthermore, antigen-driven B cell humoral responses have now been demonstrated within muscle in PM, as well as DM. A previous study has shown that immunoglobulin transcripts are prominent in PM muscle, with a relative abundance of CD138+ plasma cells (81). Further work has confirmed local B cell maturation occurs within myositis muscle in both subtypes of IIM; B cells and corresponding plasma cells were clonally expanded, had class-switched and undergone mutation (82). This suggests antigen-driven responses may play a crucial role in disease pathogenesis. The role for B-cell autoimmunity in PM and DM is further emphasised by the production of autoantibodies against specific intra-cellular protein targets, including the aminoacyl-tRNA-synthetases, signal recognition particle and Mi-2.

Non-immune mechanisms

It has been suggested that non-immune mechanisms may play a role in disease pathogenesis. The rationale being some patients can have minimal inflammatory cell infiltrate but marked structural changes on muscle biopsy with significant weakness. In a mouse model, over-expression of class I MHC molecules has been shown to mediate skeletal muscle fibre damage, even in the absence of T and B

cells (83). Similarly, in human myositis, induction of class I MHC in muscle fibres occurs before mononuclear cell infiltration (84, 85). Recent data suggests continuous upregulation of MHC class I on muscle fibers leads to an endoplasmic reticulum (ER) stress response. A potential mechanism has been proposed whereby ER overload leads to activation of nuclear factor kappa-B pathway combined with the ER unfolded protein response, which in turn promotes pro-inflammatory cytokine activity, cell death and muscle fibre necrosis (86).

1.8.2 Immunogenetics

Evidence for a genetic basis for IIM has largely accumulated through candidate gene studies, although a familial predisposition is suggested from case reports of familial clustering (87). Candidate gene studies have confirmed that HLA-DRB1*0301 and HLA-DQA1*0501 are definite risk factors for the development of Caucasian IIM (9, 87-89). These alleles form part of the conserved, ancestral Caucasian haplotype, A1-B8-Cw7-DRB1*0301-DQA1*0501. Recent studies have confirmed IIM in different ethnic groups are associated with different HLA-DR and DQ alleles (8, 89-91).

Major histocompatibility complex (MHC) differences exist between the traditional clinical PM and DM sub-types, where HLA-DRB1*07 confers risk for DM but is protective for PM (9). The 8.1 ancestral MHC haplotype, (HLA-B*08-DRB1*03-DQA1*05-DQB1*02) is associated with adult and juvenile IIM (9, 10), where the strongest signal appears to come from the HLA class I region (89, 92). Furthermore, TNF-308A single nucleotide polymorphisms (SNP) are associated with IIM, but only due to strong linkage disequilibrium with HLA-B*08, thus forming part of an extended 8.1 haplotype (92). Clinical phenotype is more strongly associated with myositis autoantibodies than with traditional clinical sub-type in adult Caucasian IIM (9, 89). HLA class II haplotype (DRB1-DQA1-DQB1) is in turn associated with antibody status. For example in adults, the DRB1*07-DQA1*02-DQB1*02 haplotype is strongly associated with anti-Mi-2, whereas the 8.1 haplotype is strongly associated with anti-Jo-1 autoantibodies (see also Section 1.6.2) (9).

1.8.3 Environmental factors

Environmental triggers may also influence the development of myositis. Evidence implicating potential environmental factors includes the observation that seasonal patterns are associated with the development of specific IIM-serological syndromes e.g. anti-Jo-1 positive patients have been shown to develop disease in the Spring

(93, 94). In a recent study, seasonal birth patterns in myositis subgroups suggested an aetiological role of early environmental exposures (95). In particular, birth distributions appeared to have a stronger seasonal association in JDM, suggesting a potential role for specific perinatal exposure.

There is evidence to suggest an aetiological role of ultra-violet (UV) light in the pathogenesis of autoimmune CTD. From a clinical perspective SLE and DM are associated with photosensitive rashes, and disease flares can be induced by sunlight exposure. Studies have demonstrated that UV-B light induces apoptosis of keratinocytes, which leads to the redistribution of certain intracellular proteins that are classically recognised by SLE-associated autoantibodies (96-98). In particular, autoantigens were shown to be clustered into two distinct populations, either in surface blebs or larger surface bodies on apoptotic cells (96). Apoptotic blebs contained cytoplasmic components including fragmented endoplasmic reticulum and the Ro52 protein. Apoptotic bodies contained nuclear associated autoantigens, nucleosomal DNA, Ro60, La, and the small nuclear ribonucleoproteins. These findings implicate apoptotic cells as an important potential source of concentrated or modified autoantigens that may drive autoantibody production and skin disease in SLE (97). Similar to SLE, there is evidence to suggest an aetiological role of UV-light in the pathogenesis of DM. Firstly, the incidence of DM cases has been shown to be higher in countries with a latitudinal gradient towards the equator (99). Secondly, surface UV-radiation intensity has been shown to be associated with the relative frequency of DM cases, especially those with anti-Mi-2 autoantibodies (classically associated with hallmark cutaneous DM lesions, see section 1.6.4.1). Collectively, this data provides insight into the possible aetiological mechanisms of the development of DM.

1.8.4 Autoantibodies and targeted autoantigens

The identification and characterisation of autoantibodies is an important cornerstone in the diagnosis of autoimmune CTD (4, 100, 101). Autoantibodies target nuclear or cytoplasmic intra-cellular components and specific profiles are associated with clinical phenotypes within the CTD disease spectrum. Autoimmunity is characterised by the loss of tolerance to self-proteins or autoantigens. In recent years there have been several attractive paradigms proposed to explain why certain seemingly ubiquitously expressed proteins are selectively targeted by autoantibodies. Autoantigens appear to be critical partners in driving autoreactivity, unified by their susceptibility to cleavage by certain apoptotic substrates. Studies have

demonstrated the protease granzyme B, an enzyme released by cytotoxic T cells, generates a unique form of apoptosis that leads to the cleavage of certain intra-cellular proteins into novel fragments. These fragments become clustered and may trigger autoreactive responses. This mechanism has been strongly implicated in the pathogenesis of several autoimmune diseases including SLE, SSc and IIM (96, 97, 102-106). Furthermore, this apoptotic trigger is likely to occur in specific pro-immune microenvironments and there is now emerging evidence to suggest that specific autoantigens are upregulated in different cell types and lesional tissues, particularly in IIM (107, 108).

1.8.4.1 Autoantibodies in myositis

Previous studies have shown that autoantibodies are detected in approximately 50-60% of IIM patients depending on the detection methods used (46, 109, 110). The standard techniques have included immunofluorescence on human-epithelial cell lines (HEp-2 cells), generic or antigen-specific ELISA tests, immunodiffusion and counter-immunoelectrophoresis. More specific techniques including immunoblotting and in particular protein radioimmunoprecipitation (IPP) have the advantage of being able to detect novel autoantigen targets. The major advantage of IPP is that it identifies those autoantibodies that target conformational epitopes. In addition, novel targets are amenable to identification using a proteomic approach. The identification and characterisation of novel autoantibody subsets in IIM in this study are based on the method of IPP.

Autoantibodies in IIM can be divided broadly into two groups, myositis-specific autoantibodies (MSAs) or myositis-associated autoantibodies (MAAs). MAAs are found in myositis overlap syndromes, in particular SSc, MCTD and SLE. In general, MSAs are unique to PM and DM, and the previously described specificities are outlined below. See Tables 1 and 2, and Figure 1.

Anti-synthetase autoantibodies

Autoantibodies against the cytoplasmic aminoacyl-tRNA synthetase enzymes (ARS) are the most frequently detected in adult patients with myositis. The first to be described and most common autoantigenic target is Jo-1 (histidyl tRNA-synthetase) (111), which is found in approximately 20% of IIM patients (112, 113) . Autoantibodies to six other ARS molecules; PL-7 (threonyl), PL-12 (alanyl), EJ (glycyl), OJ (isoleucyl), KS (asparaginyl) and Ha (tyrosyl) have been described collectively in approximately 20% of patients with IIM (frequency of patients with each non-Jo-1 anti-ARS is between 1-5% (114-119). A further anti-ARS termed anti-Zo (phenylalanyl) has been discovered by our group and will be described in Chapter 4 (120). The ARS are a distinct group of enzymes that catalyse the binding of specific amino acids to their cognate tRNA for incorporation into growing polypeptide chains. The anti-ARS autoantibodies define the anti-synthetase syndrome, which has been described in Section 1.5.1.

Anti-SRP autoantibodies

Patients with autoantibodies to the signal recognition particle (SRP) appear to form a further distinct clinical and histopathological subset. *Reeves et al* were the first to report SRP as an autoantigen target in myositis (121). The SRP autoantigen is a ribonucleoprotein complex (proteins-7SLRNA) with the 54 kDa and 72 kDa polypeptides most frequently targeted. The cytoplasmic SRP protein recognises secretory or membrane bound proteins and regulates protein translocation through the endoplasmic reticulum. Anti-SRP autoantibodies are found in approximately 5% of patients with IIM (122, 123). Patients with anti-SRP can present with acute onset severe myopathy with significant muscle enzyme elevation and systemic features including dysphagia that can be refractory to standard treatments (123, 124).

Anti-Mi-2 autoantibodies

Anti-Mi-2 is classically detected in adult and juvenile patients with DM clinical features. Anti-Mi-2 is detected in between 5-20% of patients with DM (6, 10, 125-128). Mi-2, a nuclear helicase protein forms part of the nucleosome remodelling deacetylase (NuRD) complex, which plays a role in gene transcription (129). This autoantibody specificity is described in patients with hallmark cutaneous DM lesions including Gottron's papules, heliotrope rash, cuticular overgrowth and the V-sign / Shawl-sign. Moreover, patients may have milder muscle involvement with a lower risk of interstitial pneumonia and respond well to therapy (6, 125-127).

Anti-CADM-140 autoantibodies

A novel autoantibody specificity termed anti-CADM-140 in a Japanese cohort associated with CADM and rapidly progressive interstitial pneumonia (130). This autoantibody specificity was not seen in classic DM, PM or any other CTD cases. Using a series of molecular techniques the CADM-140 autoantigen has recently been identified as the cytoplasmic protein melanoma-differentiation associated gene 5 (MDA5) (131). MDA5 is involved in innate immune responses against viral infections (132).

Table 1: Myositis-associated autoantibodies, autoantigen targets and clinical features

Autoantibodies	Autoantigen and function	Clinical Phenotype
Anti-U1-RNP	snRNP ribonucleoprotein complex Splicing mRNA	Mixed connective tissue disease – overlap features including myositis, arthritis, sclerodactly, Raynaud's, diffuse parenchymal lung disease, pulmonary hypertension
Anti-Ro52/60	Ro ribonucleoprotein complex (proteins hYRNA) Binding to DNA and transcription factors	SLE, Sjogren's, overlap myositis, reported in association with anti-ARS autoantibodies
Anti-PM-Scl	Nucleolar multi-protein Biogenesis of ribosomes	Myositis-scleroderma overlap
Anti-U3-RNP	Nucleolar fibrillarin ribonucleoprotein Pre-ribosomal RNA processing	Myositis-scleroderma overlap
Anti-Ku	Heterodimer Ku-p350 complex DNA repair and phosphorylation of transcription factors	Myositis-scleroderma overlap

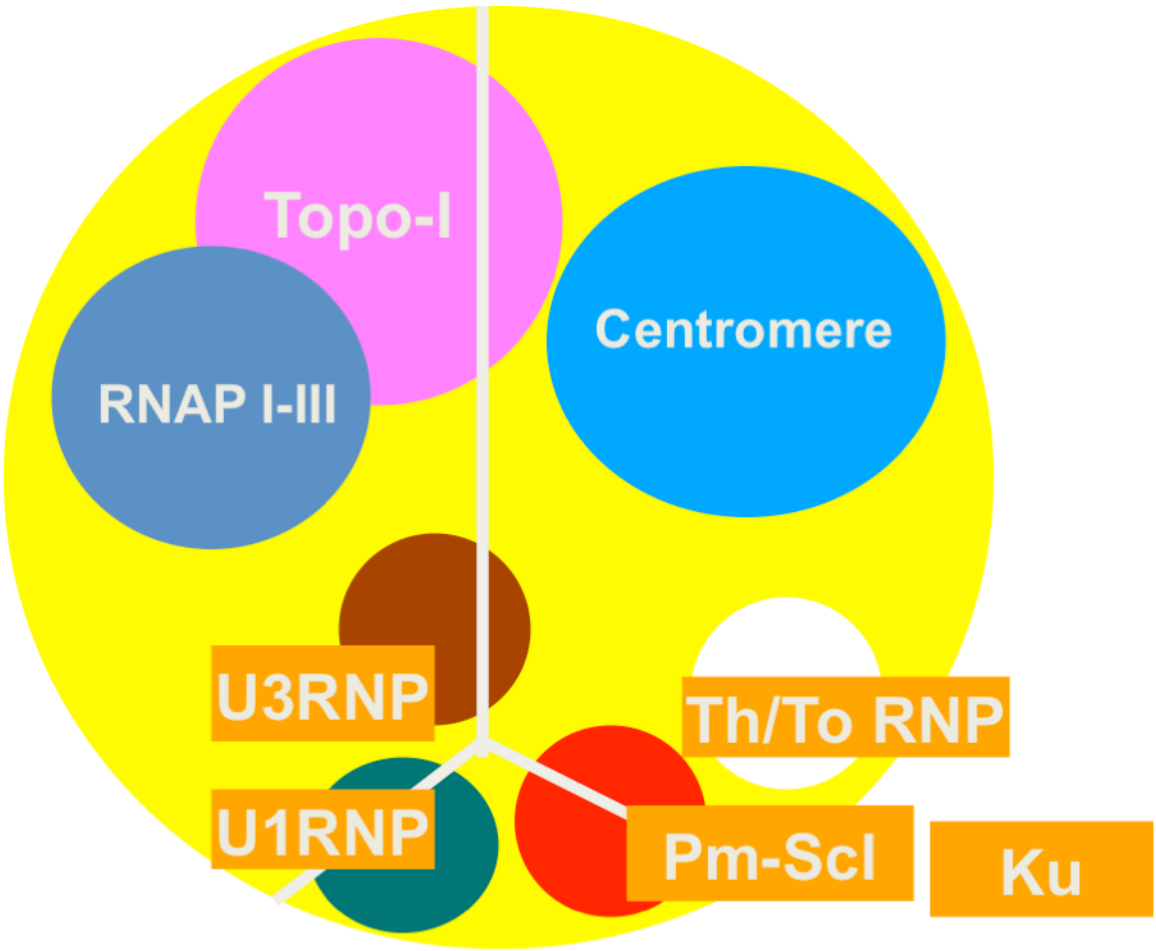
Table 2: Myositis-specific autoantibodies, autoantigens and clinical features

Autoantibodies	Autoantigen and function	Clinical Phenotype
Anti-ARS	ARS – intracytoplasmic protein synthesis (binding of specific amino acid to its cognate tRNA for incorporation into polypeptide chains)	Anti-synthetase syndrome
Anti-Jo-1	Histidyl	Myositis, mechanic's hands, Gottron's papules, arthritis, fever, Raynaud's phenomenon, high frequency of interstitial pneumonia
Anti-PL-7	Threonyl	
Anti-PL-12	Alanyl	
Anti-EJ	Glycyl	
Anti-OJ	Isoleucyl	
Anti-KS	Asparaginyl	
Anti-Ha	Tyrosyl	
Anti-SRP	SRP – intracytoplasmic protein translocation from ribosome into endoplasmic reticulum (6 polypeptides and ribonucleoprotein 7SLRNA)	Acute onset necrotizing myopathy
Anti-Mi-2	Helicase nuclear protein - transcription, remodelling of nucleosomes	Hallmark cutaneous DM, milder muscle disease
Anti-CADM-140	Intracytoplasmic MDA5 – innate immune responses against viral infections	CADM Rapidly progressive interstitial pneumonia

ARS, aminoacyl-tRNA synthetases; SRP, signal recognition particle; NuRD, nucleosome remodelling histone deacetylase; MDA5, melanoma-differentiation associated gene 5; DM, dermatomyositis; CADM, clinically-amyopathic dermatomyositis.

Figure 1: Autoantibodies in myositis and systemic sclerosis

1A: Systemic sclerosis and myositis overlap



Systemic sclerosis specific:

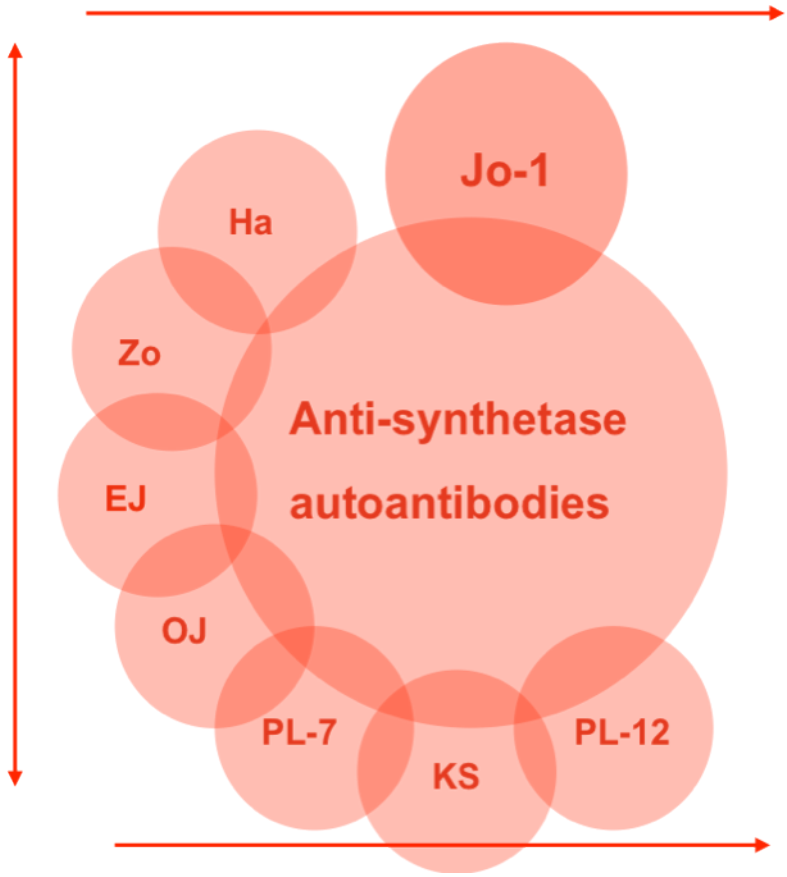
- Topo-I: Topoisomerase
- RNAP I-III: RNA polymerase I-III
- Centromere
- Th/To RNP

Myositis-associated (overlap with systemic sclerosis):

- U1RNP
- U3RNP
- PM-Scl
- Ku

Figure 1

1B: Anti-synthetase syndrome

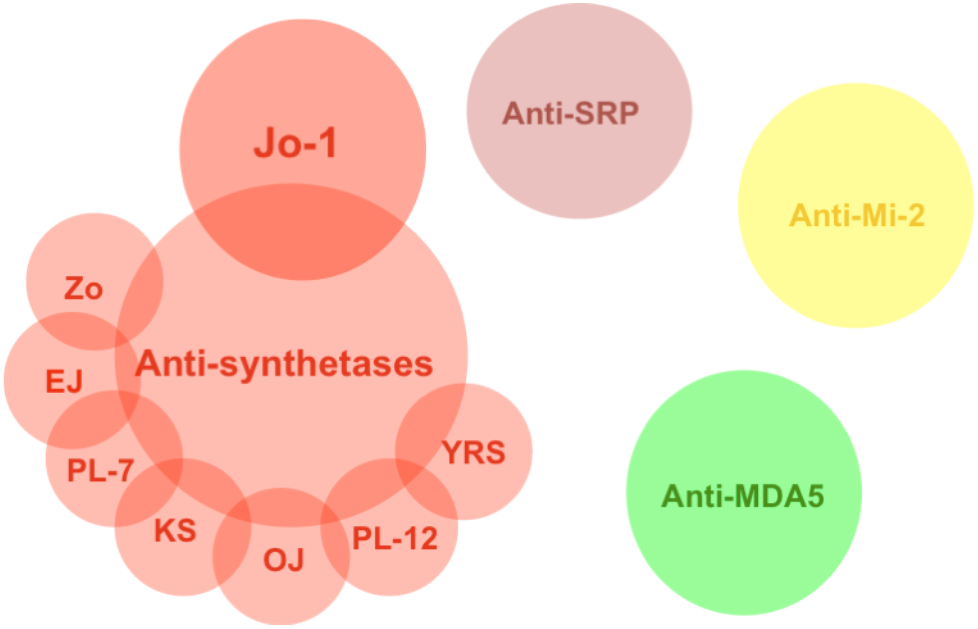


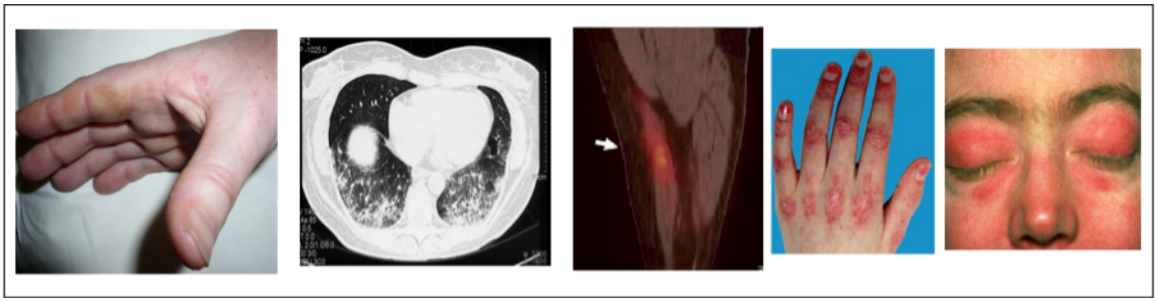
Anti-synthetase syndrome

- Myositis
- Raynaud's phenomenon
- Arthritis
- Mechanic's hands
- Gottron's lesions
- Fever
- Interstitial pneumonia

Figure 1

1C: Myositis-specific autoantibodies





- Anti-synthetase – anti-synthetase syndrome
- Anti-SRP – acute necrotising myopathy
- Anti-Mi-2 – classic DM with hallmark skin disease
- Anti-MDA5 – clinically-amyopathic DM with significant lung involvement

1.8.4.2 Autoantigens and disease mechanisms

Identification of autoimmune targets in IIM has led to further insights into pathogenesis. Perhaps the most striking feature is that groups of autoantigens with analogous cellular functions are associated with similar clinical subsets. For example, the target molecules in the anti-ARS autoantibody phenotype (anti-synthetase syndrome) and the anti-SRP autoantibody phenotype (severe necrotizing myopathy) are found in the cytoplasm with distinct functions involved in protein synthesis and translation. In contrast, the other myositis autoantigen targets are all nuclear proteins involved in gene transcription and DNA processing. Recent work has proposed the hypothesis that the dysregulated autoimmune response in myositis syndromes is driven by specific antigens in distinct tissue microenvironments. Two studies have demonstrated that certain autoantigens are enriched in lesional tissue involved in IIM (107, 108). Several myositis-specific and associated autoantigens were shown to be upregulated in myositis muscle in comparison to normal muscle especially in regenerating muscle cells. Of further interest was the observation that Mi-2 was preferentially expressed in DM muscle rather than PM muscle. The theory that distinct microenvironments may shape disease expression was emphasised with the finding that a novel conformation of Jo-1 following cleavage by granzyme B is enriched in the alveolar-epithelial layer of the lung in comparison to other tissues including muscle (108). This has lead to the suggestion that the initiating target tissue for the autoimmune response in the anti-Jo-1 syndrome is the lung with secondary attack to muscle. Further evidence implicating autoantigen driven responses has been suggested by an antigen-induced model of IIM following immunisation of congenic mice with murine Jo-1 protein (133). Antibody responses were species-specific and following immunisation mice developed the ASS clinical phenotype with muscle and lung inflammation. The potential role of other autoantigens driving autoimmunity in IIM has been highlighted by work on the chromatin remodeler Mi-2. Using a conditional transgenic mouse model, a major function of the Mi-2 protein has been elucidated (134). In particular, Mi-2 is essential for development and repair of the basal epidermis, this observation may give further insight into the pathogenic mechanisms of the anti-Mi-2 autoantibody clinical subset, of which the predominant clinical manifestation is skin disease. Further evidence to suggest a central pathogenic role of the autoantigen in IIM comes from the demonstration that certain autoantigenic ARS proteins, histidyl (Jo-1) and asparaginyl (KS) can induce leukocyte migration. In contrast, non-antigenic ARS (aspartyl-tRNA and lysyl-tRNA synthetases) were not chemotactic. The study proposed that autoantigenic ARS are over-expressed in damaged muscle

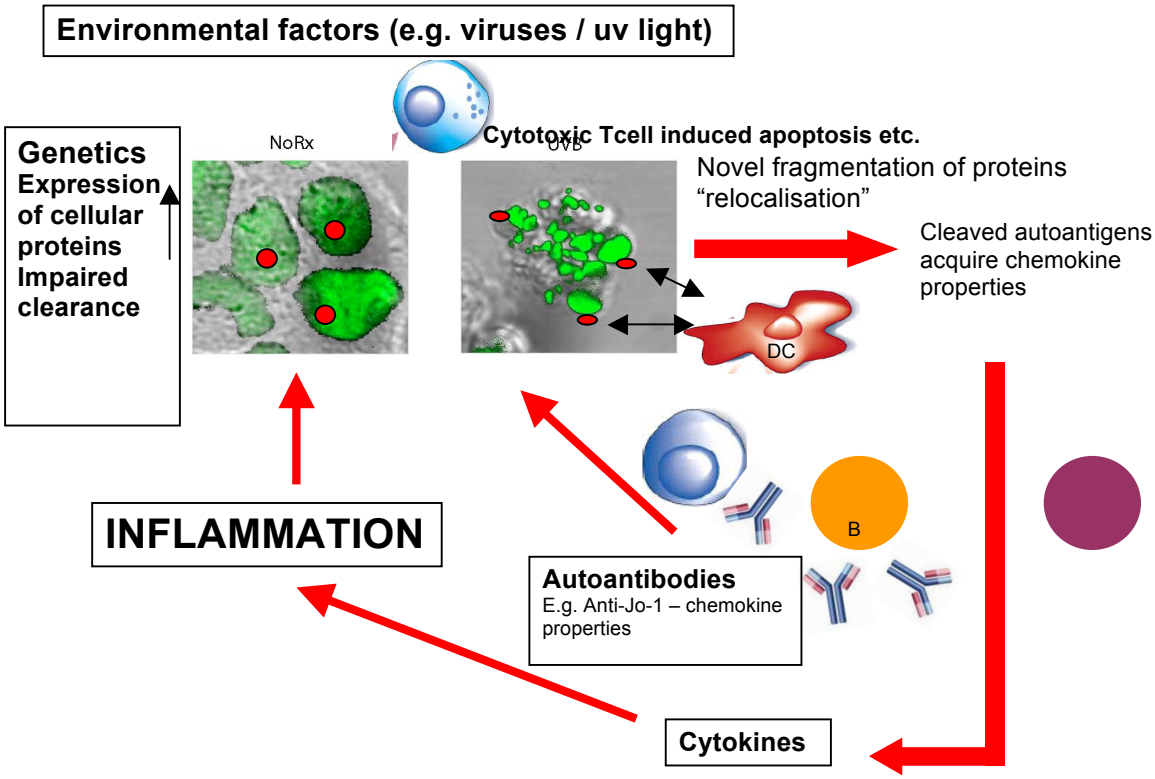
cells; their pro-inflammatory properties promote the immune response and thus the development of myositis (135). See Section 1.6.5, Figure 2.

1.8.4.3 MSAs and histopathological associations

There is also evidence to suggest that there are histopathological associations with certain MSAs, which highlights the overlap between the broad IIM subtypes. For example, patients with ASS associated myositis have distinct histopathological features with prominent perimysial inflammation, connective tissue fragmentation and perifascicular myopathic changes, in contrast to endomysial inflammation (136). Also, typical histopathological changes have been described in muscle biopsies from patients with anti-SRP autoantibodies; sparse inflammatory cell infiltrate, prominent muscle fibre necrosis and endomysial fibrosis (123, 124).

1.8.5 A proposed model for autoimmunity in IIM

Figure 2: Initiation of autoimmunity in IIM



Based on their studies, *Casciola-Rosen and Rosen* have proposed a hypothesis that the dysregulated autoimmune response in myositis syndromes is driven by novel patterns of antigen expression or conformation in distinct tissue microenvironments (107, 108, 137, 138). It is likely that a specific stimulus, for example tissue injury secondary to an infective or a toxic agent leads to a perturbed pro-immune setting in a genetically suceptible individual. In IIM, there may be three or four key initiating microenvironments i.e. muscle, lung or perhaps even skin or tumour tissue. As a result, altered antigen expression and the presentation of novel or previously unrecognised epitopes to the immune system leads to a loss of tolerance and initiation of disease. Subsequent generation of autoantibodies and generation of T cell responses may promote or propagate disease mechanisms.

1.9 Aims of study

As outlined in this chapter, in view of the clinical heterogeneity of these conditions diagnosing patients as purely DM or PM is perhaps too broad. Many investigators have proposed new sets of criteria based on updated insights. Studies suggest that clinical stratification is more appropriate according to the MSA / MAA, as the detected autoantibody and clinical phenotype are closely associated (6, 139, 140).

The hypothesis for this body of research is that adult and juvenile IIM is associated with autoantibodies that recognise specific autoantigen systems, which define patients into homogeneous clinical phenotypes. To address this hypothesis the overall aim of this thesis is to describe a comprehensive clinical and serological study of adult and juvenile IIM, including the identification of novel autoantibody subsets.

Aim 1:

To establish the prevalence and clinical features, in particular subtypes of recognised MSAs in the Royal National Hospital for Rheumatic Diseases (RNHRD) adult IIM cohort in comparison to previous published series.

Aim 2:

To investigate and characterise novel autoantibody specificities and corresponding autoantigen targets in patients recruited to the RNHRD and UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) IIM cohorts.

Aim 3:

To perform a clinical and serological study of children with JDM and JDM-overlap recruited to the UK and Ireland JDM Registry (UK JDM Cohort Study). To identify novel autoantibody-autoantigen systems, define clinical manifestations and thus markers of disease severity.

CHAPTER TWO
PATIENTS AND METHODS

2.1 Patients and sera

Subjects for the studies described in this thesis were recruited from three sources:

a) The Royal National Hospital for Rheumatic Diseases Adult Connective Tissue Disease Clinic (RNHRD)
b) The UK Adult Onset Myositis Immunogenetic Collaboration
c) The Juvenile Dermatomyositis Registry and Repository, UK and Ireland

2.1.1 Clinical diagnosis

Adult IIM patients, aged 18 years of age or older at disease onset, were recruited through the RNHRD and the UK Adult Onset Myositis Immunogenetic Collaboration, (AOMIC) (9). JDM patients were recruited to the UK and Ireland JDM National Registry and Repository (JDRR) (24). The JDRR has recruited patients with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis. All adult PM/DM and JDM cases had probable or definite disease according to *Bohan and Peter* criteria (1, 2). For adult myositis/CTD-overlap cases recruited to AOMIC, patients were included if they fulfilled all of the following: a) met published criteria for their primary CTD or mixed connective tissue disease (MCTD) (11, 12, 141, 142); b) possessed at least two of four *Bohan and Peter* criteria; (c) possessed at least one MSA/MAA. JDM-scleroderma overlap (JDM-SSc) was defined as JDM children with a history of Raynaud's phenomenon, sclerodactly and other sclerodermatous skin changes (two or more of the above features).

Demographic and clinical data were recorded at diagnosis using standardised proformas (see Section 2.1.2 and 2.1.3). Serial clinical data was also available on patients attending the RNHRD and recruited through the JDRR. Data were stored using anonymous codes onto a central database. Serum and DNA samples were taken at the time of diagnosis and stored at -20^{o}C until required.

2.1.2 Adult-onset inflammatory myopathy clinical proformas

See Chapter 2.5, Appendix I-III, pages 50-52.

2.1.3 Juvenile-onset inflammatory myopathy clinical proformas

See Chapter 2.5, Appendix IV, pages 53-60.

2.1.4 Preparation and storage of sera

Serum samples: approximately 5 ml of blood was venesected into a standard clotted sample tube. Once the blood had clotted, tubes were centrifuged at 3000 rpm for 10 min (Hereaus Labofuge 6000 Centrifuge). The serum fraction was extracted with a pipette into 500µl aliquots (adult samples) or 150µl aliquots (juvenile samples). Serum samples were stored at -20ºC.

2.1.5 Ethical approval

Collection of data and blood from patients and controls was undertaken under the regulation of the local research ethics committees and informed consent was obtained according to the Declaration of Helsinki.

2.2 Tissue culture techniques

All techniques were performed in class II tissue culture cabinets with sterile equipment. All reagents and media were purchased from Sigma, UK unless otherwise stated.

2.2.1 Preparation of tissue culture media

RPMI media

One litre of RPMI-1640 medium (with $NaHCO_3$, without L-glutamine) was supplemented with 10 ml 200 mM L-glutamine solution. Additionally, 100 ml foetal calf serum (heat inactivated) was also added for RPMI + FCS (10% foetal calf serum).

Preparation of dialysed foetal calf serum (FCS)

Two sections of dialysis tubing were cut (1 metre each, medium width), placed in a beaker of tap water and left under a running tap for 3-4 hr. The tubing was transferred to 300 ml 3% (v/w) sodium sulphide at 80ºC for 1 min, 300 ml distilled water at 60ºC for 2 min and 300 ml 0.2% (v/v) sulphuric acid at room temperature for 1 min before being rinsed in distilled water. Tubing was finally placed in 200 ml 0.02% (w/v) sodium azide and stored at 4ºC.

500 ml FCS was thawed at 37ºC overnight and PBS (2 x 3 l) was cooled at 4ºC overnight. The following day, lengths of treated dialysis tubing were rinsed in distilled water. FCS was transferred to the treated dialysis tubing, placed in 3 l cold PBS (prepared according to the manufacturer's instructions) and stirred at 4ºC for 6 hr. The PBS was replaced with a further 3 l cooled PBS and stirred at 4ºC for a

further 22 hr. Dialysed FCS was transferred to a tissue culture flask and sterile filtered through a 0.22μM filter unit. Filtered FCS was aliquoted into 50 ml tubes and stored at -20ºC until required. The sterility of the dialysed FCS was tested by adding 2 ml to 20ml RPMI + FCS and incubating at 37ºC for 48 hr.

Methionine free RPMI media (with dialysed foetal calf serum)

One hundred ml RPMI-1640 medium (modified) (with $NaHCO_3$, without methionine, cystine and L-glutamine) was supplemented with 62 μl 10% (w/v) cystine (prepared in 2M HCl and sterile filtered) and 1 ml 200mM L-Glutamine. Five ml dialysed FCS was added for methionine deficient RPMI + 5% dialysed FCS.

2.2.2 Cell culture

The non-adherent K562 cell line (human chronic myelogenous leukaemic cells) was obtained from European Collection of Cell Cultures (Salisbury, UK) (89121407). Cells were stored under liquid nitrogen at the University of Bath and transported to the cell culture facilities on dry ice.

Thawing K562 cell lines

Vials of K562 cells in 90% FCS and 10% DMSO (dimethyl sulfoxide) were transferred to dry ice and transported to the BIRD laboratory, RNHRD. Aliquots were thawed at 37ºC and added to 50 ml conical tubes containing 45 ml pre-heated RPMI + 10% FCS. Cells were incubated at 37ºC for 10 min, before being centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were discarded and the pellets were resuspended in 25 ml pre-heated RPMI + 10% FCS. Cells were transferred to 60 ml flasks containing pre-heated RPMI + 10% FCS.

K562 cells were cultured and maintained in approximately 50 ml RPMI + 10% FCS, in vented, canted, tissue culture flasks. Cells were incubated in a 5% carbon dioxide humidifier incubator set at 37 ºC.

2.3 Serological Methods

Anti-nuclear autoantibody screening was performed by indirect immunofluorescence of NOVALite HEp-2 cells INOVA Diagnostics, UK). Autoantibody specificity was further characterised by immunoprecipitation of radiolabelled cell extracts. All reagents and media were purchased from Sigma, UK unless otherwise stated.

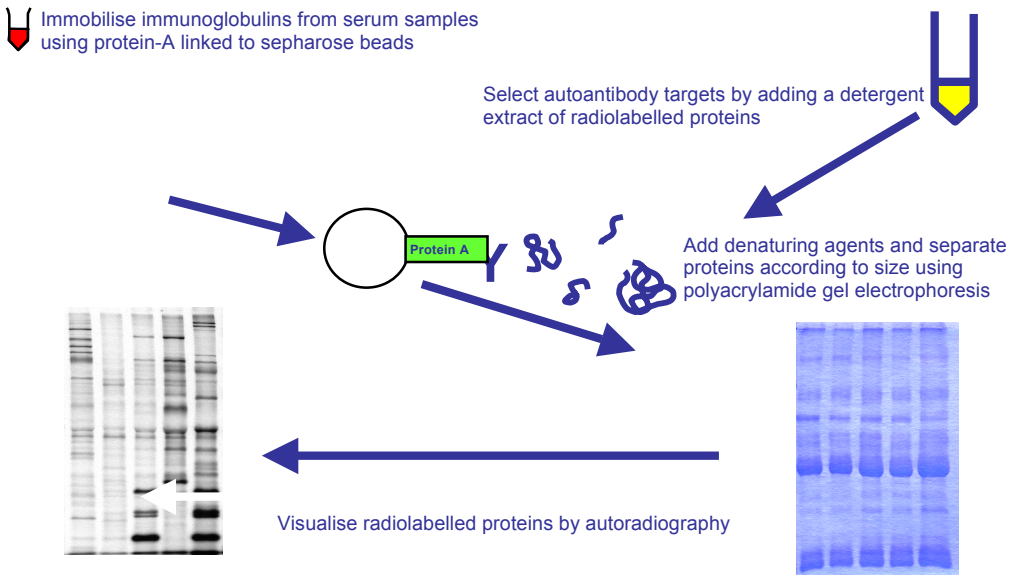
2.3.1 Indirect immunofluorescence

Serum was incubated against a fixed substrate (HEp-2 cells) to allow binding of autoantibodies to their respective antigen(s). A secondary goat polyclonal antibody conjugated to fluorescein was incubated against the substrate-antibody used at 1/150 dilution in the assay). Slides (binding site slides) were mounted and studied under a fluorescence microscope. The presence of ANA was recognised by particular patterns of fluorescence.

2.3.2 Radio immunoprecipitation (IPP) of protein targets

Serum was incubated with protein-A coated Sepharose beads to enable IgG present in the serum to bind to the protein-A via the Fc fragment. Beads were washed and supernatant removed (to remove any unbound material) and the beads incubated with radiolabelled K562 cell extract. Beads were washed further and the supernatant removed. Samples were re-suspended in Laemmli sample buffer. Finally, samples were boiled to remove and denature bound autoantigens from the Sepharose protein-A and radiolabelled proteins were separated by SDS-PAGE electrophoresis. Gels were analysed following autoradiography, protein targets were recognised by the characteristic gel fingerprint and the molecular weight of the band.

Figure 3: Protein immunoprecipitation (IPP)



Preparation of the of K562 cell extract with Methionine410

K562 culture supernatants from 6 x 150 ml flasks were transferred by pipette to 8 x 50 ml conical tubes. These were centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were sterilely removed and the pellets were pooled and re-suspended in 2 conical tubes containing 40 ml methionine free medium (without dFCS). Cells were centrifuged at 2000 rpm for 5 min and the supernatants sterilely removed. Pellets were re-suspended in 2 conical tubes each containing 40 ml methionine free medium (with FCS). A cell count (1/10 dilution) was completed on each tube. 120 x 10^6 cells were added to 4 x 120 ml flasks and the flasks were topped up to 120 ml with methionine free medium (with dFCS). Cells were incubated at 37°C for 40 min. Two hundred and fifty µl radioactive methionine (Perkin-Elmer, UK EasyTag L-^{35}S-Methionine, 5mCi (185 MBq) Stabilised Aqueous Solution) was added to each flask. The flasks were gently mixed and left at 37°C overnight. Culture supernatants were poured into 8 x conical tubes and were centrifuged at 2000 rpm for 5 min. The supernatants were discarded and the pellets pooled in 1 x conical tube containing 40 ml ice-cold Tris Buffered Saline (TBS) (10 mM Tris-HCl pH 7.4, 150 mM NaCl). A cell count was completed prior to centrifugation at 2000 rpm for 5 min. Supernatant was discarded and the cells were re-suspended in ice-cold immunoprecipitation (IPP) buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl 0.1% v/v Igepal) at a concentration of 12 x 10^6 cells / ml. The extract was sonicated using a Soniprep 150 MSE sonicator over iced-water in 7.5 ml batches (amplitude 4.5, 1 min on, 20 sec off x 4 or until no viscous lumps remained). Sonicated extract was pooled into a fresh 50 ml conical tube and then aliquoted into 1.5 ml tubes. Extract was centrifuged at 120000 rpm for 20 min at 1°C (MSE Hawk 15/05). Supernatants were pooled, transferred to fresh tubes 1.5 ml tubes (1 ml aliquots); snap frozen in dry-ice and ethanol and stored at -80°C. Any radioactive contamination was cleaned up using lipsol and appropriate standard operating procedures.

Preparation of protein-A Sepharose beads

Protein-A Sepharose beads were washed and hydrated in 40 ml IPP buffer by rotation at room temperature for 30 min. Hydrated Sepharose beads were centrifuged at 1100 rpm for 1 min (Heraeus Labofuge 6000). Supernatant was discarded and the pelleted beads were re-washed in 40 ml IPP buffer. Samples were re-centrifuged at 1100 rpm for 1 min, the supernatant was discarded and beads were re-suspended in IPP buffer at a concentration of 2 mg / ml.

Immobilisation of Antibodies

For each sample, 10 µl patient sera was mixed with 2 mg hydrated, washed protein-A Sepharose beads in 500 µl IPP buffer at room temperature for 30 min with end-over-end rotation. Autoantibody-coated Sepharose beads were pelleted at 2000 rpm for 1 min (MSE Hawk 15/05). Supernatant was discarded and the beads re-suspended in 1 ml ice-cold IPP buffer. The beads were washed a further 3 times by pelleting at 2000 rpm for 1 min, removing the supernatant and re-suspending in 1 ml ice-cold IPP buffer. After pelleting a final time at 2000 rpm for 1 min, the supernatant was discarded and the beads were resuspended in 380 µl ice-cold IPP buffer

Immunoprecipitation of Antigen

Radiolabelled K562 cell extract was thawed and 120 µl was added to each sample. Samples were mixed with end-over-end rotation at 4°C for 2 hr. Samples were centrifuged at 2000 rpm for 1 min and the supernatant discarded. Beads were washed 3 times in IPP (as outlined in the paragraph above) and once in 1 ml ice cold TBS. Beads were pelleted a final time at 2000 rpm for 1 min, the supernatant was discarded and beads were re-suspended in 50 µl SDS Laemmli sample buffer. Samples were vortexed briefly and stored at -80°C until required.

2.3.3 SDS polyacrylamide gel electrophoresis

Assembly of apparatus

The Maxi gel electrophoresis equipment (Hoefer, UK) was assembled according to manufacturer's instructions. Glass plates were washed with water, dried and cleaned with methanol. One ml thick spacers separated Glass plates and 15 well combs were used.

Preparation of gels

A 10% resolving gel was used as the standard for this procedure (see Chapter 2, Appendix V for gel recipes). Resolving gel mixture was poured into the assembled gel apparatus up to 2 cm from the top of the glass plates. Water saturated butan-2-ol (approximately 300 µl) was layered onto the top of each gel. Gels were left for at least 15 min to polymerise prior to being rinsed with distilled water. The components for a 5% stacking gel were mixed and layered on top of the resolving gel to the top of the glass plates. Teeth combs were inserted to approximately 1 cm from the top of the resolving gel. The stacking gel was left to polymerise for approximately 15 min prior to the combs being removed and the wells rinsed with distilled water.

Sample preparation

Immunoprecipitated samples were taken out of the -80°C freezer to thaw. Samples were placed into the heating block and boiled at 95°C for 4 min, removed and briefly vortexed. Samples were centrifuged at 2000 rpm for 1 min (MSE Hawk 15/05) to pellet the beads.

Gel loading and electrophoresis

Wells were loaded with 25 µl supernatant per sample. On each gel, 1 well was reserved for 15 µl pre-stained molecular weight marker (Biorad, UK). When not all the wells were used samples were spaced evenly across the gel. Once loaded, the gel apparatus was fully assembled according manufacture's instructions and transferred to the buffer chamber along with a cooling coil. Four L running buffer (25 mM Tris-base, pH7.4 190 mM Glycine and 0.1% w/v SDS) was added to the lower chamber and 500 ml running buffer was added to the upper chamber. A constant current of 60 mA was run until the dye from the sample buffer and markers had passed through the stacking gel layer (approximately 1 hr). The current was then increased to 120 mA run until the dye had passed through the resolving gel (approximately 3 hr).

Gel fixing and drying

Gels were removed from the glass plates and the stack layers discarded. The resolving gels were soaked in 0.5M sodium salicylate (enhancer) for 15 min in shallow trays. Salicylate solution was discarded; gels were rinsed in distilled water and placed in gel fix solution (methanol:water:glacial acid (4.5 :4.5:1 ratio)) for 30 min on a rocking platform. The fixative was drained off and the gels transferred onto blotting paper. The gels and blotting paper were placed on a gel dryer (Rapidry gel dryer ATTA Electrophoresis) and covered with cling-film. Gels were dried at 70°C for 70 min.

Autoradiography

Labelled proteins were analysed by autoradiography. In a dark room, dried gels were placed into X-ray cassettes and overlaid with Kodax Biomax film (mat side down). Cassettes were stored in a -80°C freezer for 1-4 weeks (time stored was dependant on the age of the radioactive preparation and the immunoreactivity of the autoantigen target). Development of films was undertaken manually in the dark room. Films were placed in 47 ml Kodax developer solution mixed with 460 ml distilled water for 2 min (until bands were exposed). Films were rinsed in distilled

water and placed into 47 ml Kodax fixer solution mixed with 460 ml distilled water for 5 min. Films were immersed in distilled water again and then left to dry at room temperature.

See Chapter 2, Appendix VI, Table 3: summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets.

2.3.4 Immunodepletion

See *2.3.2* for basic IPP methods and reagents. This technique was used to establish whether autoantibodies that recognised a novel autoantigen of the same molecular weight (in specific patient cohorts) were targeting the same protein specificity. In summary, radiolabelled cell extracts were immunoprecipitated with prototype sera (sera known to contain the novel autoantibody specificity): Serum was incubated with protein-A bound Sepharose beads and incubated with radiolabelled K562 cell extract. On this occasion, the pelleted beads bound to the autoantibody-autoantigen complex were removed and the supernatant (unbound cell extract) preserved. This procedure was repeated to ensure complete depletion of the target antigen from the cell extracts. Reference depleted cell extracts were then used for further immunoprecipitations with other autoantibody positive sera.

In detail, for each sample, duplicate tubes each containing 10 mg hydrated protein-A Sepharose beads (prepared as Section 2.3.2) and 50 µl reference sera in 1 ml IPP buffer were mixed with end-over-end rotation at room temperature for 30 min. Autoantibody-coated Sepharose beads were washed 4 x in IPP buffer (see section 2.3.2). After the beads were pelleted for the final time, supernatants were discarded. One tube for each sample (A) was placed on ice, whilst 150 µl [^{35}S]-methionine-labelled K562 cell extract and 380 µl IPP buffer was added to the remaining tube (B). Tube B was mixed with end-over-end rotation at 4°C for 2 hr after which the supernatant was transferred to tube A. This was mixed at 4°C for a further 2 hr. The supernatant from tube A was transferred to a fresh tube (C) and stored at -80°C. Immunoprecipitations using positive sera and either 120µl control [^{35}S]-methionine-labelled cell extract or the immunodepleted supernatants (C) were completed and subject to SDS-PAGE electrophoresis as described in Section 2.3.2.

2.3.5 Immunoprecipitation Western Blotting

Immunoprecipitation Western Blotting (IPP-WB) was used to determine the identify of specific autoantigens recognised by autoantibodies. Autoantigens were selected from patient's sera by immunoprecipitation and SDS-PAGE. Proteins were transferred to nitrocellulose membrane by immunoblotting. Membranes were incubated in a protein rich solution to block any remaining non-specific binding sites and probed with commercial antibody to a specific protein. Membranes were incubated with an appropriate secondary labelled anti-immunoglobulin followed by a conjugated staining substrate.

Preparation of unlabelled K562 cell extract

K562 cultures were grown to confluence in 6 flasks each containing 150 ml complete RPMI with FCS (see section 2.2.1). Supernatants were transferred to 8 x 50 ml conical tubes and centrifuged at 2000 rpm for 5 min (Heraeus Labofuge 6000). Supernatants were removed and the pellets resuspended in 2 x conical tubes each containing 40 ml ice-cold TBS. A cell count (1/10 dilution) was completed on each tube. Cells were centrifuged at 2000 rpm for 5 min, the supernatants were discarded and the pellets combined in a single conical tube containing ice-cold IPP buffer at a concentration of 15 x 10^6 cells / ml. Extract was sonicated using a Soniprep 150 MSE sonicator over iced-water in 7.5 ml batches (amplitude 4.5, 1 min on, 20 sec off x 4 or until no viscous lumps remained). Sonicated extract was pooled into a fresh 50 ml conical tube and aliquoted into 1.5 ml tubes. Extract was centrifuged at 120000 rpm for 20 min at 1°C (MSE Hawk 15/05). Supernatants were pooled, transferred to fresh 1.5 ml tubes (1.2 ml aliquots); snap frozen in dry-ice and ethanol and stored at -80°C.

Antigen preparation for IPP WB

See 2.3.2 for IPP methods and reagents. Samples, each containing 40 µl serum and 2 mg hydrated protein-A Sepharose beads (prepared as Section 2.3.2) in 500 ml IPP buffer were mixed with end-over-end rotation at room temperature for 30 min. Autoantibody-coated Sepharose beads were centrifuged at 2000 rpm for 1 min (MSE Hawk 15/05), supernatant was discarded and beads re-suspended in 1 ml 0.2M tri-ethanolamine pH 8.1. Beads were washed a second time (centrifuged at 2000 for 1 min and re-suspended in tri-ethanolamine) prior to being centrifuged again and re-suspended in 5 mM bis-(sulphosuccinimidyl)-suberate cross-linker (Perbio, UK) in 1 ml 0.2M tri-ethanolamine. Beads were mixed with end-over-end rotation at room temperature for 30 min and centrifuged at 2000 rpm for 1 min.

Beads were re-suspended in 1 ml 50 mM Tris-Cl pH 7.5 and mixed with end-over-end rotation at room temperature for 15 min. Beads were centrifuged at 2000 rpm for 1 min and washed three times in PBS and two times in ice-cold IPP buffer. Beads were incubated in 1 ml unlabelled K562 cell extract with end-over-end rotation for 1 hr at 4°C, centrifuged at 2000 rpm for 1 min and resuspended in 1 ml fresh K562 unlabelled cell extract. Samples were incubated for a further 1 hr at 4°C with end-over-end rotation. Beads were washed 4 times in ice-cold IPP buffer and once in TBS. Samples were finally re-suspended in 80 µl SDS sample buffer and stored at -80°C until required.

SDS-PAGE and transfer

Proteins were fractionated using by 10% SDS-PAGE as per standard methods (see 2.3.3), however samples were loaded at 80 µl per lane. During the electrophoresis, blotting sheets and nitrocellulose membranes were soaked in Transfer Buffer pH7.4 (40mM tris, 20 mM Sodium acetate, 2mM EDTA, 20% v/v methanol and 0.1% w/v SDS). Once electrophoresis had been completed, gels were placed in the immunoblotting transfer apparatus according to manufacturers' instructions. 'Transfer sandwiches' were prepared as scotchbrite, blotting paper, nitrocellulose membrane, gel, blotting paper and scotchbrite. The surfaces of the 'transfer sandwiches' were smoothed over to remove any air-bubbles. The 'transfer sandwiches' were placed into the tank apparatus that was filled with Transfer Buffer. A cooler system was inserted and proteins were transferred from the gels to the nitrocellulose membranes using a constant current at 220 mA for 4.5 hr. Nitrocellulose sheets were removed and the corners were marked. Membranes were rinsed in distilled water and were Ponceau stained for 5 min. Membranes were rinsed again for 4 x 15 sec in distilled water and air-dried overnight. Membranes were stored in a sealed container at 4°C until required.

Immunoblotting and staining

Membranes were washed in PBS-0.05%Tween for 5 min and blocked for 90 min in block solution (5% milk powder in PBS-0.05%Tween, pH7.4). Membranes were incubated with commercial primary antibody (diluted in block solution accordingly to manufacturer's instructions) for 90 min. Membranes were washed 5 x 5 min in PBS-0.05%Tween and re-incubated with the corresponding secondary antibody (commercial anti-IgG) at a 1:20000 dilution for 90 min. Membranes were washed 5 x 5 min in PBS-0.05%Tween and finally incubated in substrate stock for 10-20 min. Membranes were rinsed with distilled water for 5 min and air-dried overnight.

2.4 Statistical analysis

Clinical and autoantibody associations were derived from 2×2 contingency tables using the chi-squared test with Yates' continuity correction, or two-tailed Fisher's exact test where individual cells valued five or less. Where significant, data were expressed as odds ratios (OR) with exact 95% confidence intervals (CI). Where data was not normally distributed the Mann-Whitney-U test was used to compare continuous data. Median values (inter-quartile ranges) were expressed where appropriate. *P* values (P_{corr}) were adjusted using the Bonferroni correction when comparing clinical associations. Uncorrected probabilities are presented for possible clinico-serological associations. P values <0.05 were considered significant. *SPSS for Windows (version 14)* was used to perform statistical analysis.

2.5 Appendix

I. RNHRD Clinical proforma (Page 50)

II. AOMIC Clinical Proforma (Page 51)

III. Autocure Clinical Proforma (Page 52)

IV. JDRR clinical Proforma (forms 1 and 2) (Pages 53-60)

V. Reagents and gel recipes (Pages 61-63)

VI. Table 3: Summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets (Page 64)

Permission to reproduce AOMIC, Autocure and JDRR proformas granted by:

*Dr. Hector Chinoy, Consultant Senior Lecturer, University of Manchester

**Prof. Lucy Wedderburn, Scientific Director, JDRR, University College London

NOVEL AUTOANTIBODIES IN MYOSITIS STUDY

PATIENT DETAILS:

Name: Hospital no:

Sex: Ethnic origin:

D.O.B: Age at disease onset:

CLINICAL DETAILS:

1. Presentation
   - Cutaneous disease only [ ]
   - Muscle disease only [ ]
   - Both [ ]

2. Development of myositis* in relation to cutaneous features (please tick):
   - Never [ ]
   - 3 months [ ]
   - 6 months [ ]
   - 12 months [ ]
   - Other – please specify [ ] months

3. Creatinine kinase at disease onset (if available) [ ]

4. Maximum creatinine kinase during follow up [ ]

5. Skin lesions (please tick):
   - Heliotrope rash [ ]
   - Gottron's sign [ ]
     - Extensor aspect of fingers [ ]
     - Over elbows or knee [ ]
   - Periungal / nailfold erythema [ ]
   - V-sign rash [ ]
   - Shawl-sign rash [ ]
   - Mechanic's hands [ ]
   - Calcinosis [ ]

6. Other features (please tick):
   - Pyrexia [ ]
   - Weight loss [ ]
   - Dysphagia [ ]
   - Raynaud's phenomenon [ ]
   - Arthritis [ ]
   - Cancer [ ]
   - Interstitial lung disease [ ]
     - Subtype (if known) – NSIP [ ]
       UIP [ ]
       Organising pneumonia [ ]

7. Other information e.g. type of cancer, extent of calcinosis, treatment::

* myositis: weakness / myopathic EMG / myositis on imaging or biopsy

MYOSITIS GENETIC STUDY
CLINICAL & LABORATORY PROFORMA

PATIENT DETAILS *(complete or ring)*

NAME:

……..

Sex: M / F Hospital No.....

Ethnic origin: Caucasian/ Negroid / Asian / Oriental
Mixed (specify.....)
Other (specify.....)

Current age:.....

Age at myositis onset:……

MYOSITIS DIAGNOSTIC GROUP *(tick or ring)*

Polymyositis **(PM)** [ ]
Dermatomyositis **(DM)** [ ]
PM as part of other **CTD** [ ]

(Specify: SLE / SSc / MCTD / Overlap / Undifferentiated)

SYMPTOM / LESION INDEX - *We will only include **definite** PM/DM, according to the diagnostic criteria highlighted, so please tick which abnormalities ever present:*

1. Skin Lesions
- Heliotrope rash [ ]
- Gottron's sign [ ]
- Violaceous rash on elbows/knees [ ]

2. Systemic inflammatory signs [ ]

3. Non-destructive arthropathy [ ]

4. Muscle-specific antibody [ ]

5. Elevated skeletal muscle enzymes [ ]

6. Myalgia at rest or on contraction [ ]

7. Proximal muscle weakness [ ]

8. Myopathic EMG [ ]

9. Myositis on biopsy [ ]

If patient has lung fibrosis as part of their PM, tick below.
Lung fibrosis (clinically and/or on PFTs) [ ]

DIAGNOSTIC CRITERIA FOR PM/DM

1) Skin lesions:
   - Heliotrope rash (violaceous erythema on upper eyelids),
   - Gottron's sign (violaceous keratotic papules on extensor aspects of fingers,
   - Violacious raised rash over elbows or knees.
2) Systemic inflammatory signs (elevated ESR/CRP, pyrexias, weight loss etc)
3) Non-destructive arthritis
4) Muscle specific antibodies, e.g Anti Jo-1 etc
5) Elevated skeletal muscle enzymes
6) Myalgia at rest or with contraction
7) Proximal muscle weakness
8) Myopathic EMG
9) Myositis on muscle biopsy.

For diagnosis of **definite** PM:
At least four from item list 2 – 9.

For diagnosis of **definite** DM:
At least one feature from item 1), plus at least four from item list 2 – 9.

(From Tanimoto et al. Classification criteria for PM/DM. J. Rheumatol 1995, 22: 668-674)

UNDERLYING MALIGNANCY - *If your patient has PM/DM, in association with a proven malignancy, could you please specify site & tissue type if known*

.....……..

.....

Patient's Consultant:.....

Hospital:.....

Date form completed:……

Signature:……

RGC/2000

MANCHESTER 1824

The University of Manchester

STUDY ID.....

AUTOCURE MYOSITIS
CLINICAL & LABORATORY PROFORMA

1. CENTRE & PATIENT DETAILS *(complete / attach sticker):*

HOSPITAL *(Name/Code)*:..... .....

PATIENT*(Name)*:.....

Gender *(ring)*: **M / F**

NHS/Hospital N°:.....

Ethnicity: **Caucasian/ Afro-Carrib/ African/ Asian/ Oriental**
Mixed *(specify)*:.....
Other *(specify)*:.....

Date of birth:.....

Month/Year of myositis onset:.....

Month/Year of myositis diagnosis:.....

Ht (cm)..... **Wt (kg)**..... **Ever smoked?: YES / NO**

2. MYOSITIS DIAGNOSTIC GROUP *(please tick one of a-d):*

a) Polymyositis	**(PM)**	[ ]
b) Dermatomyositis	**(DM)**	[ ]
c) Amyopathic Dermatomyositis	**(ADM)**	[ ]
d) Inclusion Body Myositis **(please complete IBM-specific form)**	**(IBM)**	[ ]
ADDITIONALLY, if overlap with CTD: **SLE/ Sjögren's/ MCTD/ SSc/ RA** *(ring)* other *(specify)*:.....	**(Overlap)**	[ ]

3. CASE VERIFICATION *(please tick all that apply):*

1. **Proximal muscle weakness** [ ]
2. **Myositis on biopsy** [ ]
3. **Elevated skeletal muscle enzymes** [ ]
4. **Myopathic EMG** [ ]
5. **Characteristic skin lesions** [ ]

4. OTHER CLINICAL SIGNS *(please tick):*

- **Heliotrope rash** [ ]
- **Gottron's papules** [ ]
- **Violaceous rash on elbows/knees** [ ]
- **V sign** [ ]
- **Shawl sign** [ ]
- **Periungal erythema** [ ]
- **Mechanics' hands** [ ]
- **Raynaud's** [ ]
- **Arthritis** [ ]
- **Dysphagia** [ ]
- **Arrhythmias** [ ]

JV/RGC/HC/2009

5. AUTO-ANTIBODY DETECTION *(please ring):*

Myositis specific/associated autoantibody **YES / NO**

Specify:..... .....

Non myositis-specific antibodies **YES / NO**

Specify:.....

6. INTERSTITIAL LUNG DISEASE *(please ring)*

(attributable to myositis) **YES / NO**

Proven by *(ring)*: **PFTs / CXR / HRCT**

Month/year of diagnosis: .....

7. MALIGNANCY: *(regardless of whether you consider the myositis to be cancer-associated)*

Month/year of cancer diagnosis:.....

Site/tissue type:.....

8. ENVIRONMENTAL FACTORS: *(please ring/complete)*

?Statin / fibrate use prior to onset of myositis YES / NO

(specify drug if known):.....

Patient's view of what triggered myositis

(specify):.....

Exposures: Asbestos/Silica/Fibreglass/Solvents/Coaldust

other *(specify)*:.....

Patient's Physician *(print)*:.....

Date form completed *(print)*:.....

Bohan and Peter Diagnostic Criteria for Adult PM/DM[1]

1) Symmetrical weakness of limb-girdle muscles and/or anterior neck flexors.
2) Muscle biopsy evidence typical of myositis.
3) Elevation of serum skeletal muscle enzymes.
4) Typical EMG features of myositis.
5) Typical DM rash, including:
   - Heliotrope rash; violaceous erythema on upper eyelids.
   - Gottron's papules; violaceous keratotic papules on extensor aspects of fingers.
   - Violaceous rash over extensor aspects elbows/knees.

Exclusion criteria: Absence of other forms of myopathy, e.g. inclusion body, metabolic, inherited or infectious forms.

Probable / definite **PM:** at least 3 of items 1-4 +ive.
Probable / definite **DM:** item 5 & at least 2 of items 1-4 +ive.

Adult PM/DM	Must fulfill probable or definite *Bohan & Peter criteria (see highlighted box).*
Adult PM/DM-Overlap	*Must fulfill criteria for their main CTD[2-6] and at least 2 of items 1-4 and have at least one myositis specific /associated autoantibody.*
JDM	*Attending physician's diagnosis accepted (tick which criteria were satisfied for diagnosis).*
Amyopathic DM	*Must satisfy Sontheimer's criteria[7].*
IBM	*Must satisfy Grigg's criteria[8].*

References:

1. PM/DM, Bohan A, Peter JB. N Engl J Med 1975;292: 344-7; **2. SLE,** Tan EM et al. Arthritis Rheum 1982;25:1271-7; Hochberg MC. Arthritis Rheum 1997;40:1725; **3. SSc,** Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum 1980;3:581–90; **4. RA,** Arnett FC et al. Arthritis Rheum 1988;31:315-4; **5. Sjögren's,** Vitali C et al. Arthritis Rheum 1993;36:340-7; **6. MCTD,** Alarcon-Segovia D. Clin Dermatol 994;12:309-16; **7. Amyopathic,** Sontheimer RD. J Am Acad Dermatol 2002; 46:626-36; **8. IBM,** Griggs RC et al. Ann Neurol 1995;38:705-13.

JUVENILE DERMATOMYOSITIS COHORT BIOMARKER STUDYAND REPOSITORY

FORM 1 – INITIAL PRESENTATION INFORMATION

Please tick

Has consent/assent form for the registry and repository been completed? ☐

A. DEMOGRAPHICS:

DATE OF VISIT: **PATIENT REF NO:**

Patient status*: Male/Female

DOB:

Ethnicity: 01 02 03 04 05 06 07 08 09
(Please see guidelines for codes. If 04 or 09, please specify)

Postcode:

Occupation of parents:

*1=Initial visit/diagnosis at GOS/MDX
2=GOS/MDX primary institution for JDM care after previous diagnosis elsewhere
3=Seen for occasional care/shared care only (i.e. GOS/MDX not primary centre)
4=Other (please specify)

B. HISTORY UP TO DIAGNOSIS:

1. Background data DIAGNOSIS:_____

y):
yyyy

I. General symptoms:	Absent	Present	Not known
Rash	0	1	9
Weakness	0	1	9
Fever	0	1	9
Alopecia	0	1	9
Weight loss	0	1	9
Fatigue	0	1	9
Mouth ulcers	0	1	9
Headache	0	1	9
Irritability	0	1	9
Raynauds	0	1	9

II. Musculoskeletal:	Absent	Present	Not known
Myalgia	0	1	9
Joint pain	0	1	9
Jt stiffness	0	1	9
Jt swelling	0	1	9
Dyspnoea	0	1	9
Dysphonia	0	1	9
Dysphagia	0	1	9

At diagnosis if available

Height: _____cm

Weight: _____kg

BP: _____/_____

			Not known
			9
			9
Diarrhoea	0	1	9
Melaena	0	1	9
Haematuria	0	1	9
Facial/body swelling	0	1	9

3. Past medical history	*Absent*	*Present*	*Please specify where details known/relevant*
Specific viral infection	0	1	
Other autoimmune disease	0	1	
Other significant diagnosis	0	1	
Trauma/injury	0	1	
Immunisation in last 6/12	0	1	
School absence	0	1	
Stopped PE/Sport	0	1	
Respiratory problems	0	1	
GI problems	0	1	
Neurological involvement	0	1	
Medication related problems	0	1	
FH of autoimmune disease	0	1	

C. EXAMINATION FINDINGS AT DIAGNOSIS:

I. Skin:	*Absent*	*Present*	*Not known*
Gottrons papules	0	1	9
Ulceration	0	1	9
Oedema	0	1	9
Calcinosis	0	1	9
Nailfold changes	0	1	9
Lipoatrophy	0	1	9
Other:			
Specify/describe:_____			

	Absent	*Present*
II. Arthritis	0	1
III. Muscle weakness	0	1
IV. Other:	0	1
Specify_____		

D. CLINICAL COURSE SINCE DIAGNOSIS: (***Tick this box and go to form 2 if patient recruited at diagnosis***)

I. General symptoms:	*Absent*	*Present*	*Not known*
Rash	0	1	9
Weakness	0	1	9
Fever	0	1	9
Alopecia	0	1	9
Weight loss	0	1	9
Fatigue	0	1	9
Mouth ulcers	0	1	9
Headache	0	1	9
Irritability	0	1	9
Raynauds	0	1	9

II. Musculoskeletal:	*Absent*	*Present*	*Not known*
Myalgia	0	1	9
Joint pain	0	1	9
Jt stiffness	0	1	9
Jt swelling	0	1	9
Dyspnoea	0	1	9
Dysphonia	0	1	9
Dysphagia	0	1	9

III. Systemic features:	*Absent*	*Present*	*Not known*
Chest pain	0	1	9
Abdo pain	0	1	9
Diarrhoea	0	1	9
Melaena	0	1	9
Haematuria	0	1	9
Facial/body swelling	0	1	9
Calcinosis	0	1	9

E. INVESTIGATION RESULTS AT DIAGNOSIS:

0=normal, 1=abnormal, 9=not done: Please tick the appropriate box for each test result.

		RESULT				FURTHER INFORMATION Please record any significant information
INVESTIGATION	**DATE**	**0**	**1**	**9**	**Value**	
Haematology						
Hb						
WBC						
Platelets						
WBC Diff: Neut						
WBC Diff: Lymph						
ESR						
Biochemistry						
CRP						
Urea						
Creatinine						
CK						
LDH						
ALT						
AST						
Albumin						
Other (specify):						
Immunology						
RF						
ANA						
ENA						
dsDNA						
Other antibodies (specify):						
C3						
C4						
IgG						
IgA						
IgM						
ASOT						
Radiology						
MRI						
Muscle Ultrasound						
DEXA: T Score						
DEXA: Z Score						
Lung Function						
VC						
FEVI						
DLCO						
Other						
EMG						
Muscle Biopsy						
Skin Biopsy						
Barium Swallow						
Echocardiogram						
Urine Dipstick						
Other (specify):						

F. THERAPY INFORMATION SINCE DIAGNOSIS

I. CORTICOSTEROIDS	*No*	*Yes*	*Not known*
Oral steroids	0	1	9
IV Steroids	0	1	9

II. DMARDs	*No*	*Yes*	*Not Known*
Methotrexate	0	1	9
Cyclosporin A	0	1	9
Azathioprine	0	1	9
Cyclophosphamide	0	1	9
Hydroxychloroquine	0	1	9
IV-Immunoglobulin	0	1	9
Etanercept	0	1	9
Infliximab	0	1	9
Other (specify):	0	1	

_____

III. NSAIDs	*No*	*Yes*	*Not known*
Naproxen	0	1	9
Other (specify):	0	1	

_____

IV. OTHER	*No*	*Yes*	*Not known*
Calcium/Vit D	0	1	9
Folic acid	0	1	9
Plasmaphoresis	0	1	9
Other (specify):	0	1	

_____

G. PHYSIOTHERAPY/OCCUPATIONAL THERAPY

	No	*Yes*	*Not known*
Dry land	0	1	9
Hydrotherapy	0	1	9
Splinting	0	1	9
Other	0	1	Specify: _____

H. OTHER INFORMATION

No 0 *Yes* 1

Specify:

Signature:	**Position:**
Print name:	**Date:**

JUVENILE DERMATOMYOSITIS COHORT BIOMARKER STUDY AND REPOSITORY

FORM 2: CLINIC VISIT FORM

Patient Reference No: Date of visit:

A. HISTORY SINCE LAST CLINIC VISIT
(or in last 3 months – whichever is most recent)

1. Clinical History

I. General Symptoms	*Absent*	*Present*
Rash	0	1
Weakness	0	1
Fever	0	1
Alopecia	0	1
Weight loss	0	1
Fatigue	0	1
Mouth ulcers	0	1
Headache	0	1
Irritability	0	1
Raynauds	0	1

II. Musculoskeletal	*Absent*	*Present*
Myalgia	0	1
Joint Pain	0	1
Joint stiffness	0	1
Joint Swelling	0	1
Dyspnoea	0	1
Dysphonia	0	1
Dysphagia	0	1

III. Systemic Features	*Absent*	*Present*
Chest pain	0	1
Abdo pain	0	1
Diarrhoea	0	1
Melaena	0	1
Haematuria	0	1
Facial/body swelling	0	1

2. Measurements

Current height:_____cm

Current weight:_____Kg

BP:_____

3. Family History

	No	*Yes*
Any new family history in the last 3 months:	0	1

Specify:

B. EXAMINATION FINDINGS

I. Skin	*Absent*	*Present*
Gottrons papules	0	1
Ulceration	0	1
Lipoatrophy	0	1
Oedema	0	1
Nailfold changes	0	1
Calcinosis	0	1
Other	0	1

Specify/describe:_____

II. Distribution of rash	*Absent*	*Present*
Periorbital	0	1
Periungal	0	1
Trunk	0	1
Small joints	0	1
Large joints	0	1
Other	0	1

Specify:_____

III. Joints

	Absent	Present	Please specify which joints are affected
Arthritis	0	1	_____
Pain on motion	0	1	_____
Joints with limited ROM	0	1	_____
Contractures	0	1	_____

IV. Manual Muscle Testing

Neck flexors	0	1	2	3	4	5	9
Shoulder abductors	0	1	2	3	4	5	9
Elbow flexors	0	1	2	3	4	5	9
Wrist Extensors	0	1	2	3	4	5	9
Hip Extensors	0	1	2	3	4	5	9
Hip Abductors	0	1	2	3	4	5	9
Knee Extensors	0	1	2	3	4	5	9
Ankle Dorsiflexors	0	1	2	3	4	5	9

*0=no muscle action, 1=flicker of muscle action, 2=muscle action with gravity counterbalance, 3=muscle action against gravity, 4=muscle action against gravity with some resistance, 5=full muscle strength, (9=not done)

V. Oedema

	Absent	Present
Periorbital/facial	0	1
Limb	0	1
Trunk	0	1

VI. Abdomen

	Absent	Present
Abdominal masses	0	1
Tenderness	0	1
Hepatomegaly	0	1
Splenomegaly	0	1

VII. Respiration

Please circle one: 0 1 2 3 4 5

0=normal, 1=SOBE, 2=tachypnoea, 3=accessory muscle use, 4=requires oxygen, 5=ventilated

VIII. Other

	Absent	Present	Please specify
Calcinosis	0	1	_____
Eyes (glaucoma/cataracts)	0	1	_____
Other	0	1	_____

IX. Physician Global Assessment

Disease least active 0 _____ 10 Disease most active

C. PHYSIOTHERAPY/OCCUPATIONAL THERAPY

	No	Yes	
Dry Land	0	1	Daily/weekly/other
Hydrotherapy	0	1	Daily/weekly/other
CMAS done Today	0	1	Score: _____/53
CHAQ done today	0	1	Score: _____/3
CHQ done today	0	1	Physical score:_____ Psychological score:_____
Parents VAS	0	1	Score: _____
Splinting	0	1	Specify:_____
Other	0	1	Specify:_____

D. INVESTIGATION RESULTS: 0=normal, 1=abnormal, 9=not done - tick the appropriate box for each result

		RESULT				FURTHER INFORMATION Please record any significant information
INVESTIGATION	**DATE**	**0**	**1**	**9**	**VALUE**	
Haematology						
Hb						
WBC						
Platelets						
WBC Diff: Neut						
WBC Diff: Lymph						
ESR						
Biochemistry						
CRP						
Urea						
Creatinine						
CK						
LDH						
ALT						
AST						
Albumin						
Other (specify):						
Immunology						
RF						
ANA						
ENA						
dsDNA						
Other antibodies (specify):						
C3						
C4						
IgG						
IgA						
IgM						
ASOT						
Radiology						
MRI						
Muscle Ultrasound						
DEXA: T Score						
DEXA: Z Score						
Lung Function						
VC						
FEVI						
DLCO						
Other						
EMG						
Muscle Biopsy						
Skin Biopsy						
Barium Swallow						
Echocardiogram						
Urine Dipstick						
Other (specify):						

E. THERAPY INFORMATION (since last visit)

I. Corticosteroids

	No	Yes	Current Dose	Reducing Dose?
Oral Steroids	0	1	_____ _____mg/day	Yes No
IV Steroids	0	1		

II. DMARDs

	No	Yes	Dose and type administered	Dates if started or stopped in the last 3 months
Methotrexate	0	1		
Cyclosporin A	0	1		
Azathioprine	0	1		
Cyclophosphamide	0	1		
Hydroxychloroquine	0	1		
IV-IG	0	1		
Plasmophoresis	0	1		
Etanercept	0	1		
Infliximab	0	1		

III. NSAIDs

	No	Yes	Dose on day attending clinic
Ibuprofen	0	1	
Naproxen	0	1	
Other	0	1	
Specify:_____			

IV. Other Drugs

	No	Yes	Dose on day attending clinic
Calcium/Vitamin D	0	1	
Folic acid	0	1	
Gastroprotectant	0	1	
Vasodilator	0	1	
Anti-HT	0	1	
Other	0	1	
Specify:_____			

F. RESEARCH INVESTIGATION SAMPLES

Were the following samples taken today:	*No*	*Yes*
Blood:		
Lab white bottle (hep)	0	1
Clotted bottle	0	1

G. OTHER INFORMATION *No* *Yes*

Specify:

Signature:	Position:
Print Name:	Date:

V. Reagents

- RPMI x1 liquid (Sigma)
- 10% FCS or FBS (Sigma)
- L-glutamine, 200mM (Sigma)
- Methionine free medium; solid (Sigma)
- L-leucine; solid (Sigma)
- L-lysine; solid (Sigma)
- Sodium bicarbonate (Tissue culture grade, Sigma)
- S^{35} methionine (Amersham Biosciences (GE Healthcare) AG 1095 5mCi)
- Protein A or protein G sepharose beads (Sigma)
- Immunoprecipitation (IPP) buffer

(10mM Tris/HCl, pH 8.0, 500mM NaCl, 0.1% v/v Igepal; previously known as Nonidet P-40)

- Tris buffered saline (TBS) buffer

(10mM Tris/HCl, pH 7.4 + 150mM NaCl)

- Sodium Phosphate (crosslinking for MS)

0.1M Sodium Phosphate pH8.1 (0.1M NaH_2PO_4 to 500ml 0.1M Na_2HPO_4 until pH=8.1)

- Tris-Cl (crosslinking stop buffer)

1.0M Tris base pH7.5

- Triethanolamine (crosslinking reaction buffer)

0.2M Triethanolamine pH8.1

- Laemmli sample Buffer (SDS-PAGE)

Sigma S3401

- Western Blot Substrate

Sigma B1911

- SDS-PAGE Marker

Biorad Kaleidoscope Marker - 161-0324

- Blocking powder (Western Blot)

5% Sainsburys semi-skimmed milk powder, pH 7.4 (with NaOH)

- PBS-Tween

PBS with 0.05% Tween

- Phosphate Buffered Saline

Sigma P4417 (1 tablet per 200 ml water)

- Western Blot Transfer Buffer

40 mM Tris, 20 mM Sodium Acetate and 2 mM EDTA
pH 7.4 with acetic acid
Trizma base (Sigma)

V. Reagents

- Glycine
- Sodium chloride (BDH)
- Conc. HCl (Sigma)
- Glacial acetic acid (Sigma)
- PBS tablets (Sigma)
- Acrylamide/bis-acrylamide, 30% solution (Sigma)
- Ammonium persulphate (Sigma)
- Kalidescope markers, broad range containing glycerol, protein and bromophenol blue (Bio-Rad)
- TEMED (Sigma)
- Sodium dodecyl sulphate (SDS), (Sigma)
- Sample buffer containing mercaptoethanol, glycerol, bromophenol blue, tris and SDS (Sigma)
- Methanol (Sigma)
- Butan-2-ol (Sigma)
- Sodium salicylate (Sigma)
- DMSO (Sigma)
- Trypan blue (Sigma)
- Conc. Sulphuric acid (BDH)
- Sodium sulphide (Sigma)
- Sodium azide (Sigma)
- pH buffer standards (Lab 3)

V. Gel recipes

Percentage acrylamide in gel mixture (volumes are per gel)

Vol (ml)	5%	8%	9%	10%	12.5%	15%
Water	11.8	14.5	13.5	12.5	10	7.5
Lower gel buffer		7.5	7.5	7.5	7.5	7.5
Upper gel buffer	5					
Acrylamide mixture	3.2	8	9	10	12.5	15
AMPS	0.1	0.21	0.175	0.15	0.11	0.09
TEMED	0.03	0.06	0.04	0.03	0.02	0.02
Total volume	~20	~30	~30	~30	~30	~30

30% (w/v) acrylamide:bisacrylamide (30:5:1)

Lower gel buffer stock: 1.5M Tris/HCl, pH 8.8 + 0.4% (w/v) SDS

Upper gel buffer stock: (*5% upper gels in this procedure) 0.5M Tris/HCl pH 6.8 + 0.4% (w/v) SDS

10% (w/v) ammonium persulphate

VI. Table 3: Summary data of molecular weights / apparent molecular weight run on 10% SDS-PAGE of recognised myositis-specific autoantigen targets

	Autoantigen	Molecular Weight (kDa)	Runs at (10% SDS-PAGE) (kDa)
Jo-1	Histidyl-tRNA synthetase	57.4	52
PL-7	Threonyl-tRNA synthetase	83.4	80
PL-12	Alanyl-tRNA synthetase	106.7	110
KS	Asparaginyl-tRNA synthetase	62.9	65
EJ	Glycyl-tRNA synthetase	83.1	75
OJ	Multi-enzyme complex		
	Leucyl-tRNA synthetase	125.0	130
	Isoleucyl-tRNA synthetase	145.0	150
	Glutaminyl-tRNA synthetase	160.0	170
Ha	Tyrosyl-tRNA synthetase	59	62
SRP	Signal recognition particle	262.3	240
Mi-2	SNF2 super-family helicase	55.7	54

CHAPTER THREE
RESULTS
RNHRD clinico-serological study of myositis-specific autoantibodies in adult-onset inflammatory myositis

3.1 Introduction

There is increasing evidence that patients with adult-onset idiopathic inflammatory myopathies (IIM); polymyositis (PM) and dermatomyositis (DM) have serological profiles within the disease spectrum (7, 46, 112, 139). Myositis specific autoantibodies (MSAs) appear to be important prognostic markers and are associated with specific clinical features.

The aim of this study, described in this chapter, was to investigate the prevalence of recognised MSAs in the Royal National Hospital for Rheumatic Diseases (RNHRD) IIM cohort in comparison to previous published series (6, 46, 112). This study also highlights the clinical features associated with the different MSAs, in particular lung disease. The work described here has been presented at the *British Society of Rheumatology 2008* and the *American College of Rheumatology 2006*:

- Gunawardena H, Betteridge Z and McHugh NJ. The prevalence and clinical associations of myositis specific and associated autoantibodies in idiopathic inflammatory myopathy (abstract). Rheumatology 2008;47(S2):ii84.
- Gunawardena H, Robinson G, Betteridge Z, North J, Carmichael C, Foley NM, McHugh NJ. Interstitial pneumonia in polymyositis and dermatomyositis (abstract). Arthritis Rheum. 2006;54:S660.

3.2 Patients and Methods

3.2.1 Patients

Patients with a diagnosis of adult IIM (age of onset 18 years or over) were identified from the RNHRD CTD database and clinics. Cases of myositis / connective tissue disease (CTD) overlap were excluded. The clinical notes were reviewed to confirm the diagnosis of PM or DM based on the *Bohan and Peter* criteria (1, 2), and clinical data were retrospectively recorded using a standardised proforma (see Chapter 2, Appendix 1). Data recorded included details on initial presentation, creatinine kinase (CK) at disease onset and maximum CK during disease course. The degree of

cutaneous involvement and other features were noted including the presence of lung disease and cancer-associated myositis.

3.2.2 Assessment of interstitial pneumonia

High resolution computerised tomography (HRCT) scans were re-reviewed under the guidance of Consultant Thoracic Radiologist (GR) and the subtype of interstitial pneumonia was categorised based on the American Thoracic Society / European Respiratory Society classification (32).

3.2.3 Serological Methods

ANA-Immunofluorescence (IIF)

IIF was previously performed by standard methods (PO and JD) using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK). The ANA IIF results were reviewed from the clinical notes and RNHRD / Bath Institute for Rheumatic Diseases serological database. The ANA titre and pattern was recorded.

Protein Radio-immunoprecipitation (IPP)

Sera stored at -20^{o}C were thawed at room temperature. IPP from K562 cell extracts was performed as previously described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [^{35}S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

3.2.4 Ethical approval

All patients gave fully informed written consent to participate and provide biological samples according to the Declaration of Helsinki under local ethical committee regulations.

3.3 Results

Fifty patients with adult-onset IIM were identified from the RNHRD CTD database. The serological profiles and clinical associations are described below:

3.3.1 Serological profiles

Following IPP, autoantibody specificity was categorised into recognised MSAs, myositis-associated autoantibodies (MAAs), unidentified or negative. The frequency of recognised MSAs in this cohort of 50 patients was: anti-aminoacyl-tRNA synthetases (anti-ARS) 28%, anti-SRP 8% and anti-Mi-2 4%. Fourteen% of cases had MAAs (8% anti-PM-Scl, 4% anti-Ro, 2% anti-Ku). Twenty-two% had autoantibodies that appeared to recognise distinctive autoantigen bands (see Chapter 4). Twenty-four% of patients sera were either negative or precipitated non-specific bands by IPP. See Table 4 for summary and Figure 4.

3.3.2 Clinical associations

The main clinical features of patients with MSAs; anti-ARS, anti-SRP, anti-Mi-2 and MAAs (anti-PMScl, anti-Ku, anti-Ro) are described in Table 5. The clinical associations of the novel autoantibody specificities are described in Chapter 4. The main clinical features of anti-ARS positive patients (n=14) were arthritis 78.6%, mechanic's hands 57.1%, interstitial pneumonia 71.4% and Raynaud's phenomenon 71.4%, and 71.4% initially presented with arthritis or respiratory disease prior to myositis onset. Anti-SRP positive patients (n=4) were characterised by severe disease; including severe myopathy (median CK 11036 IU/L), with systemic features of weight loss (50%) or fever (50%), arthritis (75%) and Raynaud's phenomenon (75%) and cardiac involvement (50%). No anti-SRP patents had interstitial pneumonia. Muscle biopsies were available from two anti-SRP-positive patients demonstrating predominant muscle fibre necrosis, endomysial fibrosis and scanty inflammatory cell infiltrate. Both anti-Mi-2 positive cases had classic DM skin lesions and myositis but no other systemic organ involvement.

3.3.3 Subtypes of interstitial pneumonia

Ten (71.4%) anti-ARS-positive patients and two (29%) of MAA-positive patients had evidence of interstitial pneumonia on HRCT. No anti-Mi-2-positive, anti-SRP-positive or autoantibody-negative patients had evidence of interstitial pneumonia. As observed in previous studies (28-30, 47, 48), anti-ARS autoantibodies were a significant risk factor for interstitial pneumonia in comparison to other autoantibody subsets (anti-ARS-negative patients) (P_{uncorr}=0.02, odds ratio 2.4, 95% confidence interval 1.3-4.6). The interstitial pneumonia radiological subtypes are described in Table 6.

The main subtype of interstitial pneumonia observed in anti-ARS-positive cases was fibrotic non-specific interstitial pneumonia (NSIP), in 5 out of 10 patients. This HRCT appearance was defined by the degree of reticular changes, with predominately a subpleural and basal distribution. The fibrosing pattern was homogenous with absence of dense fibrosis i.e. no significant traction bronchiectasis or honeycombing. Three anti-ARS-positive patients (two anti-PL-7 and one with anti-PL-12 autoantibodies) had signs consistent with usual interstitial pneumonia (UIP) on HRCT, characterised by significant reticular changes including traction bronchiectasis and honeycombing. Four patients (two anti-Jo-1-positive and two anti-PM-Scl-positive cases) had HRCT features of limited NSIP i.e. predominately ground glass pattern, which is suggestive of fine fibrosis or alveolitis with a limited subpleural or basal distribution. One patient with limited NSIP had co-existent features of organising pneumonia (OP), characterised by patchy air-space consolidation, in the same distribution. See Figures 5-7 - HRCT images of corresponding interstitial pneumonia subtypes.

Table 4: Serological profiles (RNHRD IIM study)

Autoantibody	Frequency (%) RNHRD cohort (n=50)	Frequency (%) Previous studies**
Anti-ARS		
Anti-Jo-1	16	20
Anti-PL-12	2	<5
Anti-PL-7	4	<10
Anti-OJ	2	<5
Anti-KS	0	<5
Anti-EJ	0	<10
Anti-Ha	0	<1
Anti-Zo*	4	0
Anti-SRP	8	5
Anti-Mi-2	4	<10
Novel autoantibodies*		
-p155/140 kDa target	14	21
-p140 kDa target	2	0
-p40/90 kDa target	6	0
Non-specific or negative	24	n/a
MAAs		
Anti-PM-Scl	8	<10
Anti-Ro	4	<10
Anti-Ku	2	<10

***Novel autoantibodies: anti-Zo, -p155/140, -p40/90 – see Chapter 4, p140 – see Chapter 7**

**References: (112, 143)

Table 5: Clinical characteristics of anti-ARS, anti-SRP, anti-Mi-2 and MAA-positive patients

	Autoantibody				
	Anti-ARS (n=14)	**Anti-SRP (n=4)**	**Anti-Mi-2 (n=2)**	**MAAs (n=7)**	**Negative* (n=12)**
Female	79	50	100	71	100
Median age at onset (yrs)	51.5	48.5	62.5	36	62.5
Median CK at onset (IU/L)	1421	11036	4092	2347	655
Skin lesions					
Heliotrope	14	0	100	14	25
Gottron's	43	0	100	29	33
Periungal	36	25	0	29	25
V-sign	0	0	100	0	17
Shawl-sign	0	0	50	0	17
Mechanic's hands	57	0	0	43	8
Fever	57	50	0	29	8
Weight loss	36	100	0	57	8
Dysphagia	0	50	0	14	25
Raynaud's	71	75	0	71	25
Arthritis	79	75	0	57	25
Interstitial pneumonia	71	0	0	29	0
Cardiac	0	50	0	0	0
CAM	0	0	0	0	0

***Negative or non-specific unidentified bands on IPP**

Percentages are shown unless otherwise stated

Figure 4: Immunoprecipitation of myositis-specific autoantigens

Autoradiogram of 10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extracts. The reference sera used for immunoprecipitation were; Lane 1: normal serum (QC4), Lane 2: anti-Jo-1 (QC78), Lane 3: anti-PL-12 (R12501), Lane 4: anti-PL-7 (R18134), Lane 5: anti-EJ (AOMIC MM182), Lane 6: anti-KS (AOMIC MM020), Lane 7: anti-OJ (AOMIC MM63), Lane 8: anti-Zo (R21845), Lane 9: anti-SRP (R7417), Lane 10: anti-Mi-2 (R18411).

Arrows indicate molecular weights (kDa) –see Chapter 2, Appendix, Table 4 for running molecular weights of 10% SDS=PAGE.

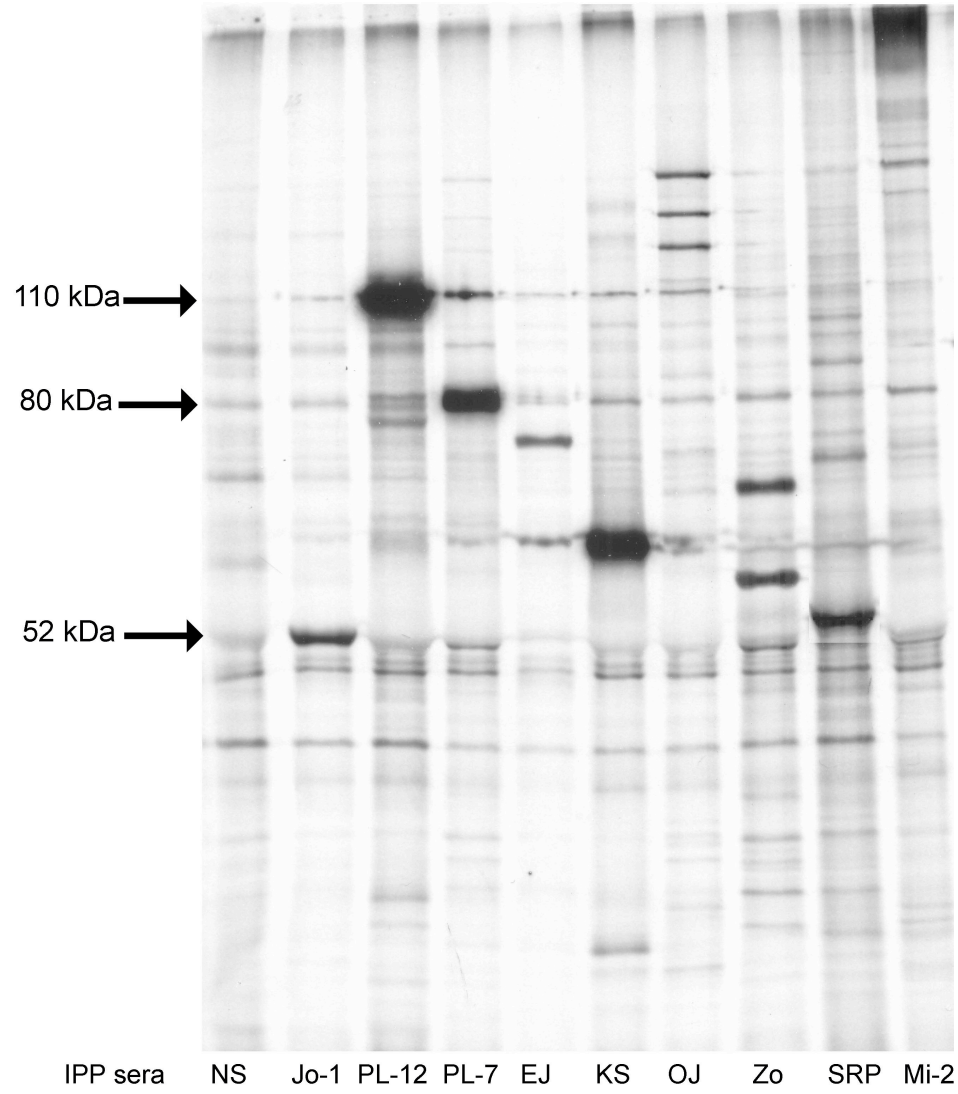


Table 6: Subtypes of interstitial pneumonia in anti-ARS-positive and MAA-positive patients (RNHRD IIM study)

Patient	Autoantibody	HRCT IP subtype
R16716	Anti-Jo-1	Fibrotic NSIP
R9435	Anti-Jo-1	Fibrotic NSIP
R13077	Anti-Jo-1	Limited NSIP
R5477	Anti-Jo-1	Fibrotic NSIP
R12707	Anti-Jo-1	Limited NSIP and OP
R18134	Anti-PL-7	UIP
R19896	Anti-PL-7	UIP
R12501	Anti-PL-12	UIP
R15222	Anti-Zo*	Fibrotic NSIP
R21845	Anti-Zo*	Fibrotic NSIP
R8125	Anti-PM-Scl	Limited NSIP
R10686	Anti-PM-Scl	Limited NSIP

***See Chapter 4**

Figure 5: Non-specific interstitial pneumonia

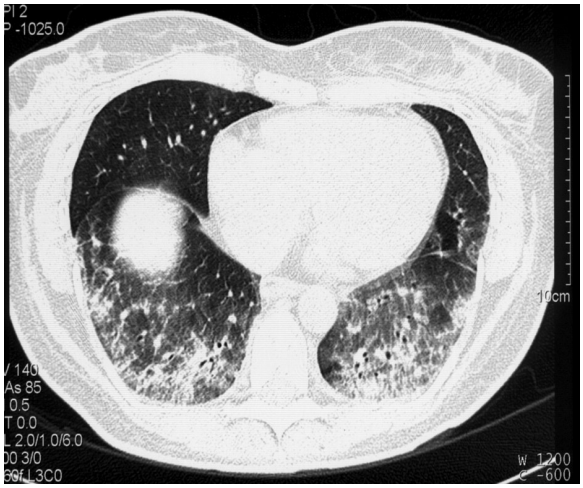


Figure 6: Usual interstitial pneumonia

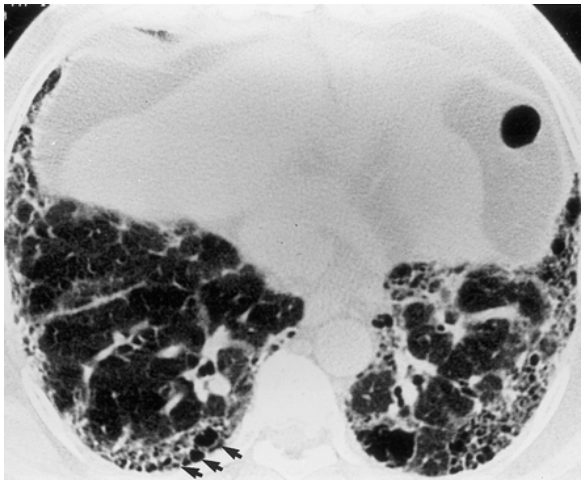


Figure 7: Organising pneumonia

3.4 Discussion

Historically, PM and DM has been defined based on broad pathological and clinical criteria (1, 2). There is now increasing evidence that these disorders characterised by a wide clinical spectrum can now be classified into more homogeneous clinico-serological syndromes (6, 46, 112, 139). Autoantibodies found exclusively in IIM, termed MSAs are directed against cytoplasmic or nuclear components involved in key regulatory processes such as protein synthesis, translocation and nuclear transcription, and have been the subject of major interest over the last few years. Unlike myositis-associated autoantibodies (MAAs) found in principally myositis-scleroderma overlap syndromes; MSAs are highly selective, mutually exclusive and are associated with characteristic clinical profiles. As described in Chapter 1; several myositis specific autoantigen systems have been described including the aminoacyl-tRNA synthetases (ARS) (111, 114-119), signal recognition particle (SRP) (121, 122) and Mi-2 (126).

Consistent with previous observations (112), in this study the most frequently detected MSA group were anti-ARS, with a lower frequency of anti-SRP and anti-Mi-2 autoantibodies. Using IPP, a number of novel autoantibody specificities were identified, which are described fully in Chapter 4.

The clinical manifestations of anti-ARS-positive patients were consistent with the anti-synthetase syndrome, with a high frequency of mechanic's hands, fever, arthritis, Raynaud's phenomenon and interstitial pneumonia. As previously described in anti-synthetase syndrome (42), the majority of patients presented with extra-muscular manifestations, in particular interstitial pneumonia, prior to their myositis onset. Clinically-amyopathic disease or milder muscle involvement is described especially in non-Jo-1 anti-ARS-positive cases (42, 144-148). In comparison to anti-ARS-negative cases, anti-ARS-positivity was a significant risk factor for the presence of interstitial pneumonia, as observed previously (6, 31, 42, 48, 144-147, 149-152). Consistent with previous findings (30, 38), NSIP was the frequent interstitial pneumonia subtype observed in anti-ARS-positive patients (including all anti-Jo-1-positives). Interestingly, anti-PL-7 or -12 positivity was associated with UIP, the radiological and histological pattern synonymous with idiopathic pulmonary fibrosis. Indeed, the predominant clinical manifestation in two of these patients was interstitial pneumonia, with one patient (anti-PL-7-positive) developing myositis approximately 10 years after the onset of lung disease and one

patient (anti-PL-12-positive) has never developed clinical myositis. In some cases, IPF may actually represent a *formes frustes* of autoimmune connective tissue disease, especially in patients with non-Jo-1 anti-ARS (150), which may not be tested for in routine clinical practice. In a study by *Matsushita et al* 25% of patients with idiopathic interstitial pneumonia were anti-ARS positive (152). At present, it remains unclear why Jo-1 is the most common autoantigenic target in ASS or why patients with anti-ARS autoantibodies have differences in their clinical presentation. In these patients, a high index of clinical suspicion including the recognition of other subtle clinical features, for example a history of arthralgia or fever, is required.

Similar to previous studies (122-124), all patients with anti-SRP autoantibodies had evidence of severe myopathy with markedly elevated CK at disease onset. In addition, patients had systemic features, cardiac involvement and arthritis but no significant skin disease, and none had evidence of IP. Therefore, identification of anti-SRP autoantibodies at disease onset has prognostic implications, identifying those patients at risk of severe muscle disease that may be refractory to standard treatments and thus require more intensive management. Anti-Mi-2 is a DM-specific-autoantibody (6, 10, 125-128), and in this study, both patients with anti-Mi-2 autoantibodies had myositis associated with classic cutaneous DM lesions with no systemic features or internal organ involvement.

In summary, the MSAs: anti-ARS, -SRP, -Mi-2, identify IIM patients into three distinct clinical phenotypes. Rather than diagnose patients as purely DM or PM, if we adopt a serological classification to define patients into clinical syndromes this may help predict outcomes, in particular, identify those patients who are at risk of severe disease, which in turn may influence treatment strategies.

3.5 Appendix

I. SUBJECTS

Code	IIF pattern	IPP result	HRCT pattern
R12707	Coarse Cytoplasmic Speckle	Jo-1	Limited NSIP/OP
R16716	Cytoplasmic Speckle	Jo-1	Fibrotic NSIP
R13077	Discrete Cytoplasmic Speckle	Jo-1	Limited NSIP
R9435	FS	Jo-1	Fibrotic NSIP
R5477	Negative	Jo-1	Fibrotic NSIP
R4480	Discrete Speckles	Jo-1	n/a
R11739	Not available	Jo-1	n/a
R8452	FS and Discrete Cytoplasmic	Jo-1	n/a
R12501	Coarse Cytoplasmic Speckle	PL-12	UIP
R18134	Fine Cytoplasmic Speckle	PL-7	UIP
R19896	FSNS and Strong Fine Cytoplasmic Speckle	PL-7	UIP
R16682	Not available	OJ	n/a
R15222	Strong Cytoplasmic Speckle	Zo*	Fibrotic NSIP
R21845	Strong Cytoplasmic Speckle	Zo*	Fibrotic NSIP
R20227	FSNS and Strong Fine Cytoplasmic Speckle	SRP	n/a
R7417	Negative	SRP	n/a
R22034	Homogeneous with Fine Cytoplasmic Speckle	SRP	n/a
R12767	FSNS	SRP	n/a
R18411	Homogeneous	Mi-2	n/a
R16125	Homogeneous	Mi-2	n/a
R8125	Nucleolar	PM-Scl	Limited NSIP
R10686	Not available	PM-Scl	Limited NSIP
R17606	Homogeneous and nucleolar	PM-Scl	n/a
R14303	FS and nucleolar	PM-Scl	n/a
R9980	Negative	Ro	n/a
R15323	DCNS	Ro	n/a

Code	IIF pattern	IPP result	HRCT pattern
R21233	Homogeneous and Fine Cytoplasmic Speckle	Non-specific bands	n/a
R18506	FS and nucleolar	Non-specific bands	n/a
R13440	FSNS	Non-specific bands	n/a
R19013	FSNS	Ku	n/a
R13902	Homogeneous and Nucleolar	Non-specific bands	n/a
R13408	Negative	Negative	n/a
R7544	FS	Negative	n/a
R21308	FSNS	Negative	n/a
R10290	Negative	Negative	n/a
R14789	Homogeneous and Coarse Cytoplasmic Speckle	Negative	n/a
R9674	Homogeneous	Negative	n/a
R21140	DCNS	Negative	n/a
R19484	FSNS and Cytoplasmic Speckle	Negative	n/a
R20125	FSNS	155 and140 kDa bands*	n/a
R9316	FSNS	155 and140 kDa bands*	n/a
R7505	DCNS	155 and140 kDa bands*	n/a
R16815	FSNS	155 and140 kDa bands*	n/a
R8315	DCNS	155 and140 kDa bands*	n/a
R13064	Homogeneous	155 and140 kDa bands*	n/a
R11248	Homogeneous	155 and140 kDa bands*	n/a
R18883	FS and Fine Cytoplasmic Speckle	140 kDa*	n/a
R16316	CS	40 and 90 kDa bands*	n/a
R11040	DCNS	40 and 90 kDa bands*	n/a
R20894	FSNS	40 and 90 kDa bands*	n/a

***See Chapter 4**

N.A. – not applicable

ANA IIF patterns - abbreviations:

C: coarse

D: diffuse

F: fine

S: speckle

NS: nucleolar sparing

II. Acknowledgements

Mrs J Dunphy (JO) and Mrs P Owen (PO) previously performed ANA IIF on sera from IIM cases as part of the RNHRD (Bath Institute for Rheumatic Diseases) Diagnostic Laboratory Service. I would like to thank them for giving me permission to include this data in this study.

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I would like to thank all the patients who have attended the RNRHD CTD clinic and given their consent to participate in these studies.

CHAPTER FOUR

RESULTS

Identification and characterisation of novel autoantigen systems in adult-onset inflammatory myositis

4.1 Introduction

To date, several myositis specific autoantibodies (MSAs) have been described including the anti-aminoacyl-tRNA synthetases (ARS) (6, 114-119), -signal recognition particle (SRP) (6, 123, 124) and -Mi-2 (6, 125-127) (as described in Chapter 1, see Table 2, for summary). Over the last few years, new MSAs have been described in adult IIM.

Sato *et al* have described an autoantibody termed anti-CADM-140 in a Japanese cohort of adult patients with clinically amyopathic dermatomyositis (CADM) (130). .As defined by Gerami *et al*, patients with CADM have hallmark cutaneous features of DM but no clinical evidence of myositis (40). Moreover, CADM patients especially those of Asian origin, appear to have an increased risk of more severe interstitial pneumonia (31, 41, 153). In this study by Sato *et al*, anti-CADM-140 autoantibodies were detected in 8 out of 42 DM patients (19%) but not in patients with PM, other CTDs or control groups. All 8 patients with anti-CADM-140 autoantibodies were clinically amyopathic. Anti-CADM-140-positive sera demonstrated a granular or reticular cytoplasmic staining pattern on immunofluorescence (IIF) using HEp-2 cells. Following radio-immunoprecipitation (IPP) using ^{35}S-methionine-labelled K562 cell extract, anti-CADM serum recognised an ~140 kDa polypeptide, which was distinguishable from other known MSAs, and immunodepletion studies confirmed that this autoantibody recognises the same target. Of interest, the frequency of severe interstitial pneumonia was significantly increased in the anti-CADM-140-positive group compared to anti-CADM-140 negatives (50% versus 6%, p=0.008) (130). Using a series of molecular techniques including a HeLa cell-derived complementary DNA library, the identity of the ~140 kDa protein has recently been identified as a RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) (131). MDA5 is involved in innate immune responses against viruses (132, 154, 155). This finding suggests that a specific viral infection in genetically susceptible individuals may play a direct role in the pathogenesis of CADM and severe interstitial pneumonia, particularly as this MSA has been detected in Japanese patients, and confirmed in a further cohort recently (156).

A further novel MSA, termed anti-p155 or anti-p155/140 has been reported in adult DM. In the study by Targoff *et al*, following IPP using HeLa cell extract, anti-p155-positive sera recognised a distinct 155 kDa protein (along with a weaker 140 kDa protein in most cases) in 8 out of 39 DM patients (21%). Anti-p155 sera revealed a nuclear speckled pattern on IIF with HEp-2 cells (143). Kaji *et al* have demonstrated what is likely to be the same autoantibody, termed anti-p155/140, in 7 out of 52 DM patients (13%) using IIP with K562 cells (157). Both studies showed that anti-p155/140 autoantibodies also appear to identify a distinct clinical phenotype with more severe skin involvement. Moreover, the most striking feature is the clear association with malignancy and Chinoy *et al* have confirmed this. The authors showed that anti-p155/140 positivity confers a significant risk for cancer-associated-myositis with a high specificity, moderate sensitivity and high negative predictive value (158). In preliminary work, the identity of the p155 protein target is consistent with transcriptional intermediary factor 1-gamma (TIF-1-γ), a nuclear transcription protein involved in cellular differentiation (159).

This chapter describes data from the RNHRD IIM cohort on anti-p155/140 autoantibodies, and also work describing two novel MSAs (termed anti-Zo and anti-SAE). The work in this chapter has been published, as listed below:

- Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Anti-synthetase syndrome: a new autoantibody to phenylalanyl transfer RNA synthetase (anti-Zo) associated with polymyositis and interstitial pneumonia. Rheumatology. 2007;46:1005-1008. (120)
- Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Identification of a novel autoantibody in dermatomyositis directed against small ubiquitin-like modifier activating enzyme. Arthritis Rheum. 2007;56:3132-3137. (160)
- Gunawardena H, North J, Wedderburn LR, Davidson JE, Betteridge ZE, Dunphy J, Chinoy H, Cooper RG, McHugh NJ. Clinical associations of anti-p155/140 autoantibodies in adult and juvenile dermatomyositis. Ann Rheum Dis. 2007;66:S68. (161)
- Betteridge ZE*, Gunawardena H*, Chinoy H, North J, Ollier WER, Cooper RG, McHugh NJ. Clinical and HLA-class II haplotype associations of autoantibodies to small ubiquitin-like modifier enzyme, a dermatomyositis-specific autoantigen target, in UK adult-onset Caucasian myositis. Ann Rheum Dis. 2009;68:1621-5. (*co-first author) (162)

4.2 Patients and Methods

4.2.1 Patients

Patients with a diagnosis of adult IIM (age of onset 18 years or over) were identified from the RNHRD CTD database and clinics. Cases of myositis / CTD overlap were excluded. The clinical notes were reviewed to confirm the diagnosis of PM or DM based on the *Bohan and Peter* criteria (1, 2), and clinical data were recorded using a standardised proforma (see Chapter 2, Appendix 1). Data recorded included details on initial presentation, creatinine kinase (CK) at disease onset and maximum CK during disease course. The degree of cutaneous involvement and other features were noted including the presence of lung disease and cancer-associated myositis. For the studies on anti-SAE autoantibodies (see Section 4.3.2) clinical data and serum samples were also available from the UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) (collaborators Dr Hector Chinoy and Dr Robert Cooper).

Sera from disease controls (150 with SSc / 40 with SLE) and 40 normal controls were also analysed (available from the RNHRD Bath Institute for Rheumatic Diseases Repository). All patients with SSc and SLE fulfilled recognised published criteria (11, 12).

4.2.2 Serological Methods

ANA Immunofluorescence (IIF)

IIF was previously performed by standard methods (PO and JD) using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).

Protein Radio-immunoprecipitation (IPP)

Sera stored at -20^{o}C were thawed at room temperature. IPP from K562 cell extracts was performed as described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [^{35}S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

Immunodepletion experiments

As described in Chapter 2, section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl [^{35}S] methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using reference sera and either 150 µl control [^{35}S] methionine-labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using [^{35}S] methionine.

Protein isolation and mass spectrometry

Autoantigen isolation and identification experiments were carried out by ZB (see Appendix 4.5 for details). Therefore, the methodology has not been described in Chapter 2, but is outlined in brief in this section. Forty µl of reference sera was mixed with 2 mg protein-A-Sepharose beads in 500 µl IPP buffer at room temperature for 30 min with end-over-end rotation. Beads were washed two times in 1 ml 0.2M triethanolamine pH 8.2 (Sigma, UK) and bound antibodies were crosslinked to the beads using 5mM bis-(sulphosuccinimidyl)-suberate (Perbio, UK) in 1 ml triethanolamine, mixing at room temperature for 30 min. The reaction was stopped with 1 ml 50mM Tris-Cl pH 7.5, mixing at room temperature for 15 min. The antibody coated Sepharose beads were washed three times in phosphate-buffered-saline and twice in IPP buffer prior to the addition of 1 ml K562 cell extract, corresponding to approximately 1 x 10^6 cells. Samples were mixed end-over-end rotation at 4°C for 1 hr. The supernatant was removed and the beads were resuspended in a further 1 ml K562 cell extract and were mixed for 1 hr at 4°C. Beads were washed four times in IPP buffer and once in TBS before being resuspended in 80 µl SDS sample buffer. After heating (95°C for 4 min), proteins were fractionated by 10% SDS-PAGE. Gels were washed 3 x 5 min in pure water, stained for 60 min using Imperial Protein Stain (Perbio, UK) and destained overnight in pure water. Unique bands were removed to a 96-well plate.

Mass Spectrometry (MS)

Samples were prepared for MS at the University of the West of England using an Ettan robotic digester (GE Biosciences). Gel pieces were destained in 50%

Methanol / 50mM Ammonium Bicarbonate, dehydrated in 70% acetonitrile, air dried and digested overnight at room temperature with 20 ng/µl modified porcine trypsin (Promega) in 20mM ammonium bicarbonate. Peptides were extracted from gel pieces to a clean plate using 50% Acetonitrile / 0.1% TFA (trifluoroacetic acid) that was then dried down. The peptides were redissolved in 50% Acetonitrile / 0.1% TFA and mixed with an equal amount of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid before being spotted on the MALDI target plate using an Ettan Robotic Spotter (GE Biosciences, UK). Peptide mass fingerprints were acquired using Waters Micromass Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF) with data acquisition and processing carried out using the MassLynx software. The database searching was performed by ProteinLynx software.

4.2.3 Ethical approval

All patients gave fully informed written consent to participate and provide biological samples according to the Declaration of Helsinki under local ethical committee regulations.

4.3 Results

4.3.1 Anti-Zo, a novel autoantibody in the anti-synthetase syndrome

Identification of anti-Zo autoantibodies to phenylalanyl tRNA synthetase

Prior to this study, seven anti-aminoacyl-tRNA (ARS) synthetase autoantibodies had been described (see Chapter 1, Section 1.6.4.1 and Table 1). As part of the work related to the study described in Chapter 3, it was noted that two IIM sera (R15222 and R21845) from the RNHRD cohort study had a strong discrete cytoplasmic speckle on IIF using HEp-2 cells, the pattern of which was suggestive of anti-ARS autoantibodies (see Figure 8). Protein IPP with the two prototype sera produced a novel pattern with two bands at approximately 60 and 70 kDa (see Figure 9A). The molecular weights of the bands immunoprecipitated did not match any of the known tRNA synthetases associated with myositis, including Jo-1, PL-7 and PL-12 (see Figure 9B) and as described for KS, EJ and OJ (see Chapter 3, Figure 4), implying that both index cases serum (R15222 and R21845) IPP pattern was due to a novel anti-ARS autoantibody. This pattern was not observed in any other disease or control groups.

In order to further characterise the anti-ARS, the corresponding autoantigen was purified and identified using SDS-PAGE and MALDI-TOF MS. Sera from either the index cases or patients with known autoantibodies to Jo-1, PL-7 or PL-12 were used

to immunoprecipitate autoantigens from a K562 cell extract. A Coomassie stained SDS-PAGE of the immunoprecipitates showed bands of expected molecular weight for the Jo-1, PL-7 and PL-12 controls (see Figure 9B) as well as 60 and 70 kDa bands from the index case. The bands were digested by trypsin, analysed by MS and SwissProt database matched. Matches required peptide coverage of over 20% and scores of approximately 12. All matches were repeated on at least two separate occasions for the controls and three separate occasions for the index case immunoprecipitates. Table 7 demonstrates the correct identification of Jo-1, PL-7 and PL-12 autoantigens in the control sera and the database matching of the autoantigens precipitated by the index case. On each of the three separate occasions, the 60 kDa band was matched to phenylalanyl tRNA synthetase alpha chain (57 kDa protein) and the 70 kDa band was matched to phenylalanyl tRNA synthetase beta chain (66 kDa protein).

Clinical features of anti-Zo autoantibodies

Index case 1 (R15222)

A 49 year old woman presented with shortness of breath; six months later she developed proximal muscle weakness, Raynaud's phenomenon, puffy fingers, mechanic's hands and arthralgia. Clinical findings demonstrated a proximal myopathy with a creatinine kinase (CK) of 9533 IU/L. Muscle biopsy confirmed a necrotising myopathy with inflammatory cells. Pulmonary function tests revealed a restrictive pattern and high-resolution computerized tomography (HRCT) showed signs of fibrotic non-specific interstitial pneumonia (NSIP).

Index case 2 (R21845)

A 34 year old man presented with shortness of breath on exertion. Over the next three months he developed Gottron's papules on the extensor aspects of his fingers, mechanic's hands, proximal myopathy with a CK of 4276 IU/L (muscle biopsy demonstrated a necrotising myopathy with inflammatory cell infiltrate), fever, Raynaud's and arthritis. HRCT thorax showed signs of fibrotic NSIP.

In summary, both patients with anti-Zo autoantibodies had typical clinical manifestations of the anti-synthetase syndrome.

Figure 8: ANA IIF – anti-Zo autoantibodies

Indirect immunofluorescence staining of Hep-2 cells after incubation with index case 1 serum (R15222) and fluorescein-labelled anti-human immunoglobulin. The cytoplasmic speckle seen at 40x magnification is shown.

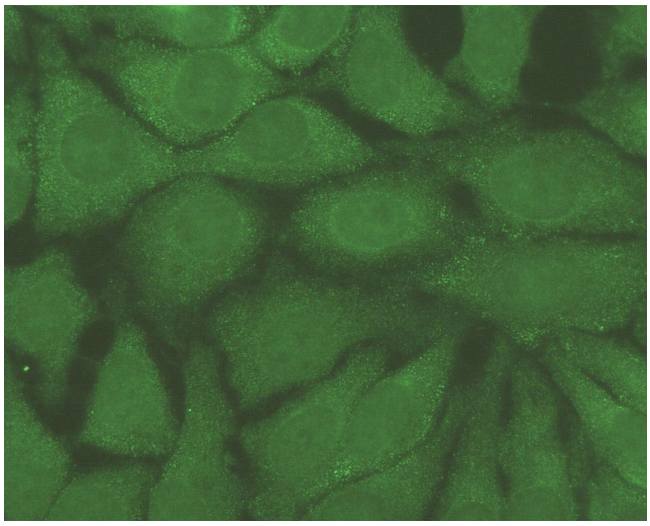


Figure 9: IPP - anti-Zo autoantibodies

A – Autoradiogram of 10% SDS-PAGE of immunoprecipitates using [^{35}S] labelled K562 cell extracts. **B** – Coomassie stained 10% SDS-PAGE of immunoprecipitates using unlabelled K562 cell extract. Sera used for immunoprecipitation include; Lane 1; control serum; Lanes 2-5, anti-synthetase sera with autoantibodies to; Lane 2 – Jo-1; Lane 3 – PL-7; Lane 4 – PL-12 and Lane 5 – index case serum, R15222 (containing autoantibodies to phenylalanyl tRNA synthetase). Molecular weight markers are indicated on the left of panel B and bands corresponding to the tRNA synthetase targets are marked.

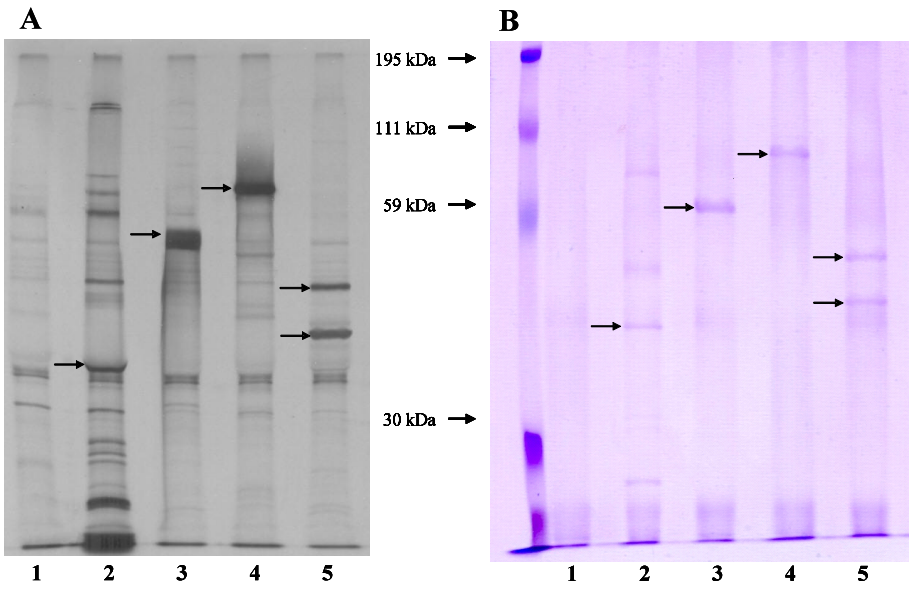


Table 7 - Results of Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) and SwissProt database matching. Comparison of peptide fragments of antigens precipitated by serum containing anti Jo-1, PL-7 and PL-12 along with peptides from the 60 kDa and 70 kDa bands precipitated by two index cases sera. Mass peptide fingerprints matched to histidyl-, threonyl- and alanyl-, phenylalanyl (alpha chain)- and phenylalanyl (beta chain)- tRNA synthetase respectively. Matches were deemed positive if the peptide coverage was over 20%, the same major theoretical and experimental peaks were present and the maximal MALDI-TOF score (using the software and database combination) was approximately 12.

Accession Number	Name	Description	[1]Mwt (Da)	[2]PI	Coverage	Score
P12081	SYH_Human	Histidyl tRNA synthetase (Jo-1)	57348	5.827	21.6%	11.7
P26639	SYTC_Human	Threonyl tRNA synthetase cytoplasmic (PL-7)	83381	6.592	32.0%	12.3
P49588	SYA_Human	Alanyl tRNA synthetase (PL-12)	106734	5.387	27.0%	11.5
Q9Y285	SYFB_Human	Phenylalanyl tRNA synthetase alpha chain	57396	7.881	29.2%	12.2
Q9NSD9	SYFB_Human	Phenylalanyl tRNA synthetase beta chain	66087	6.777	33.4%	12.3

[1]Mwt –theoretical molecular weight, [2]PI – therotical isoelectric point.

4.3.2 Anti-SAE: autoantibodies targeting small ubiquitin-like modifier activating enzyme in adult dermatomyositis

4.3.2.1 Anti-SAE: autoantibodies targeting small ubiquitin-like modifier activating enzyme in adult dermatomyositis (RNHRD study)

Identification of anti-SAE autoantibodies in index cases

The RNHRD IIM study described in Chapter 3 identified two DM patients with the same previously unidentified autoantibody pattern (R16316 and R11040). Sera from these patients gave a speckled nucleolar sparing staining pattern on IIF (R16316 serum also showed a fine cytoplasmic speckle) (data not shown) and were found to immunoprecipitate proteins of approximately 40 kDa and 90 kDa (see Figure 10) that did not correspond to any known autoantigen profile. This pattern was not observed in any other disease or control groups.

Autoantigen identification

Immunodepletion studies were completed to demonstrate that the IPP pattern seen with R16316 and R11040 were due to the precipitation of the same autoantigens. Cell extracts were depleted of antigen targets using normal, R16316 and R11040 sera before being used in further IPP using R16316 and R11040 sera. When the cell extracts were pre-depleted with normal serum, the 40 and 90 kDa antigens were still visible after IPP with each of the patient's sera (see Figure 10, Lanes 2 and 6). However, after pre-depletion with either patient's sera and removal of the corresponding targets, the 40 and 90 kDa bands were absent after subsequent IPP by either patient's sera (see Figure 10, Lanes 3-4 and 7-8). These results therefore provided good evidence that the serum from R16316 and R11040 both contained the same autoantibody specificity.

Identification of the SAE antigens

Non-radiolabelled IPP and Coomassie stained SDS-PAGE demonstrated the presence of 40 kDa and 90 kDa bands using both R16316 and R11040 serum (see Figure 11). MALDI-TOF MS and Swiss Prot analysis of the peptide fingerprints from these bands corresponded to SUMO 1 Activating Enzyme A subunit (SAE1) (38 kDa protein) for the 40 kDa band and SUMO 1 activating enzyme B subunit (SAE2) (71 kDa protein) for the 90 kDa band (see Table 8).

Clinical features of anti-SAE autoantibodies in index cases

Index Case 1 (R16316)

A 52 year old lady presented with a three month history of DM rash, including periungal erythema, V-sign rash and heliotrope rash. She had no muscle symptoms with a normal CK and electromyography (EMG). Six months later she developed proximal myopathy (CK 797 IU/L), dysphagia, and worsening skin disease with the Shawl-sign rash. Muscle biopsy confirmed necrotising myopathy with persistent inflammatory change. Screening investigations showed no sign of an underlying malignancy. HRCT showed limited peripheral lung involvement consistent with limited mild NSIP.

Index Case 2 (R11040)

A 62 year old man presented with clinical features of cutaneous DM, including a heliotrope rash and Gottron's papules. Six months later he developed proximal myositis with pharyngeal involvement. CK was 1086 IU/L and the diagnosis was confirmed following EMG study. Further investigations excluded an underlying malignancy but did show changes consistent with peripheral limited mild NSIP.

In summary, the results from this study suggest that anti-SAE autoantibodies may form a further serological subset in adult IIM with similar clinical manifestations. A further patient (R20894) was subsequently identified as anti-SAE-positive, with this finding included in the anti-SAE UK AOMIC study described below.

Table 8 – Results of MALDI-TOF MS and SwissProt database matching. Comparison of mass peptide fragments of antigens precipitated by patient 1 and 2 sera to small ubiquitin like 1 activating enzyme (SAE1) and (SAE2). Results from a total of three separate occasions (with two matches for R16316 and one match for R11040) are shown. Matches were deemed positive if the peptide coverage was over 20 percent and the same major theoretical and experimental peaks were present.

Accession Number	Name	Description	[1]Mwt (Da)	[2]PI	Coverage
Q9UBE0	ULE1A_ Human	Ubiquitin like 1 activating enzyme E1A SUMO1 (activating enzyme subunit 1)	38426	5.23	47.1% 46.2% 41.0%
Q9UBT2	ULE1B_ Human	Ubiquitin like 1 activating enzyme E1B SUMO1 (activating enzyme subunit 2)	71179	5.19	25.8% 37.8% 29.4%

[1]Mwt –theoretical molecular weight, [2]PI – therotical isoelectric point.

4.3.2.2 Anti-SAE autoantibodies in UK AOMIC cohort

The AOMIC registry has recruited UK Caucasian patients with adult-onset myositis, 18 years or over at disease onset, from hospitals around the UK. All patients had probable or definite myositis according to the *Bohan and Peter* diagnostic criteria (1, 2). A standardised proforma, including demographic and clinical data, was used throughout (see Chapter 2, Appendix). Cancer associated myositis defined as cancer occurring within 3 years of diagnosing myositis (as per the modified *Bohan and Peter* classification used in a previous study (9)) was confirmed by the local clinician caring for individual patients. Patients were investigated for interstitial pneumonia based on clinical symptoms and pulmonary function testing and where indicated HRCT thorax. Clinical data and serum samples from 266 myositis patients were available for analysis for this study. The median age of disease onset was 50 years (IQR 37-61) and 195 (73%) cases were female. One hundred and thirty one cases had DM, 124 PM and 11 myositis / CTD overlap. Sera and DNA samples were taken at the time of diagnosis and stored at -20^{o}C until required.

Identification of anti-SAE autoantibodies* in *UK AOMIC cohort

Sera from 266 myositis patients were screened for anti-SAE autoantibodies. Excluding the reference cases (R16316 and R11040) (as described in Section 4.3.2.1), nine further patients were identified as having 40 kDa and 90 kDa bands on SDS-PAGE, previously described as corresponding to SAE1 and SAE2 respectively (see Section 4.3.2.1 and Reference (160)). The presence of anti-SAE autoantibodies in these sera was confirmed by immunodepletion experiments. Immunoprecipitations on the 40kDa and 90 kDa positive sera were completed using cell extracts depleted with either the index case serum (anti-SAE), or with healthy control serum. All sera were positive for the 40 kDa and 90 kDa bands using healthy control depleted cell extract but were negative for the 40 kDa and 90 kDa bands with the index case (anti-SAE) depleted extract (see Figure 12). These results provide confirmation that the serum from the index cases and the nine additional patients contain the same autoantibody specificity, anti-SAE. Anti-SAE autoantibodies were not detected in any of the disease or normal control groups.

None of the 11 anti-SAE positive sera were found to co-immunoprecipitate any other recognised MSAs, although 5 patients did co-immunoprecipitate additional unidentified non-specific bands on IPP. IIF on anti-SAE positive sera demonstrated a fine speckled nucleolar-sparing pattern (titres ranging from 1/160 to 1/2560) in ten

patients with two sera also showing a fine cytoplasmic speckle (1/40 titre) and a homogeneous pattern (1/640 titre) in one case.

Clinical features of anti-SAE autoantibodies* in *UK AOMIC cohort

The overall frequency of anti-SAE autoantibody positive cases in the myositis cohort was 4%. Anti-SAE was found exclusively in DM patients (n=131), with a frequency of 8%. The detailed clinical features of the 11 anti-SAE positive patients are described in Table 9. Seven (64%) were female and the mean age was 62 years (IQR 54-68). Nine patients (82%) had a heliotrope rash, 9 (82%) patients had Gottron's lesions on the fingers and 7 (64%) had Gottron's over the large joints (elbow or knee). Eight out of 8 patients (100%) (data not available on 3 patients) had periungal changes. Seven out of 9 patients (78%) (data not available on 2 patients) had dysphagia, 3 of which had profound swallowing dysfunction, which required enteral feeding. Nine out of 11 patients (82%) had systemic features as defined by fever, weight loss and raised inflammatory markers. Two patients (18%) had cancer-associated myositis. Two patients (18%) had radiological evidence of ILD (limited non-specific interstitial pneumonia with less than 10% lung involvement on HRCT scored by a thoracic radiologist) but neither of these 2 patients had respiratory symptoms. Seven anti-SAE positive cases (78%) (data not available on 2 patients) presented with cutaneous DM only (no weakness and normal CK), but then went onto develop frank myositis. The median disease duration between the initial presentation of skin disease and myositis onset was 3 months (IQR 1, 6). After multiple corrections were applied, no significant clinical associations were observed when comparing anti-SAE positive patients with anti-SAE negative DM patients except for a higher frequency of periungal changes in the anti-SAE positive group (P_{corr}=0.03). In addition, data on the type of presentation i.e. skin disease first or skin / muscle disease at onset was not available on the anti-SAE negative DM group to make this comparison. The data is summarised in Table 10. This study has confirmed that SAE is an important autoantigen target in DM with specific clinical associations.

Table 9: Clinical features of patients with anti-SAE autoantibodies in UK AOMIC cohort

	Patient										
	1	2	3	4	5	6	7	8	9	10	11
Age at onset	51	62	75	46	59	75	63	52	56	78	66
Sex	F	M	F	M	F	F	M	F	M	F	F
Heliotrope	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Gottron's											
Fingers*	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Other**	No	No	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Periungal	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	~	~	~
V-sign	Yes	Yes	No	Yes	No	No	No	~	~	~	~
Shawl-sign	Yes	Yes	No	Yes	No	No	No	~	~	~	~
MH	No	No	No	No	No	No	No	~	~	~	~
Dysphagia	Yes	Yes	Yes	Yes	No	No	Yes	Yes	~	Yes	~
Systemic	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Weakness[#]	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Raised CK	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes
ILD	Yes	Yes	No	No	No	No	No	No	No	No	No
CAM	No	No	No	No	No	No	Yes	Yes	No	No	No
Arthritis	No	No	No	No	No	No	Yes	No	No	No	Yes
Presentation (months)[@]	S (6)	S (6)	S (6)	S (3)	S/M	S (2)	S (3)	S (2)	~	S/M	~

Key to Table 9:

*Gottron's lesions over the extensor aspects of fingers

**Gottron's lesions over the extensor aspects of elbows or knees

˜Clinical information not available

#Weakness – objective signs defined by manual muscle testing

MH (mechanic's hands)

ILD (interstitial lung disease) – patients asymptomatic / limited mild non-specific interstitial pneumonia on high resolution CT scan

CAM (cancer-associated myositis)

@S=presented skin disease first (number of months before myositis onset), S / M = presented with skin and muscle disease

Cases 1 and 2 (index cases R16316 and R11040 – RNHRD study)

Table 10: Comparison of selected clinical features in anti-SAE positive *vs.* anti-SAE negative dermatomyositis patients* (UK AOMIC cohort study)

	Anti-SAE positive (n=11)**	Anti-SAE negative (n=120)**
Females	64	74
Heliotrope	82	71
Gottron's papules (fingers)	82	63
Gottron's lesions (other)	64	48
Periungal erythema	100 #	46
V-sign rash	43	35
Shawl-sign rash	43	37
Dysphagia	78	43
Systemic	82	54
Weakness	100	89
Elevated CK	82	85
Interstitial pneumonia	18	18
CAM	18	16
Arthritis	18	22

*Values are the percentage of patients who had the clinical feature. **Not all patients had data on each clinical feature. CAM: cancer-associated myositis.
#P_{corr}=0.03 compared to anti-SAE negative DM patients.

Figure 10: IPP - anti-SAE autoantibodies

IPP of antigens recognised by patients R16316 and R11040. Autoradiogram of 10% SDS-PAGE of immunoprecipitates using either R16316 and R11040 serum with control [^{35}S] methionine-labelled cell extract or [^{35}S] methionine-labelled cell extract depleted with either normal sera (NS), patient R16316 sera (1) or patient R11040 sera (2). The bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated.

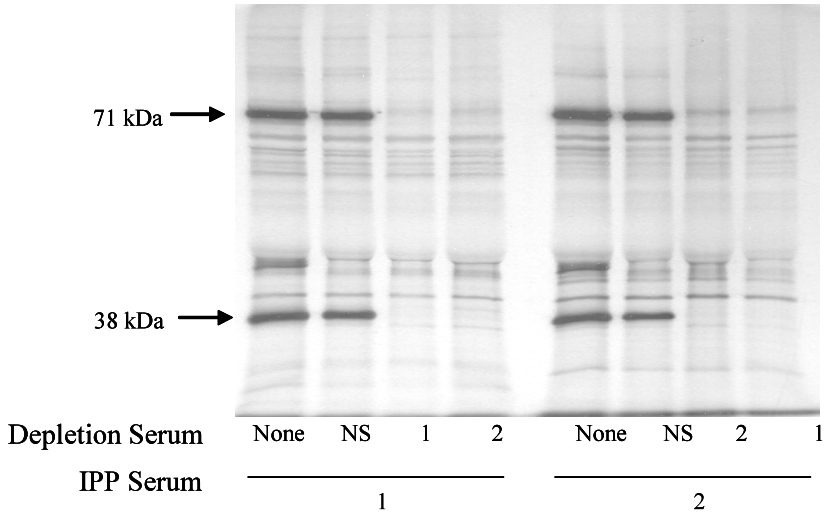


Figure 11: Coomassie stained IPP of proteins using anti-SAE antibodies

Coomassie stained 10% SDS-PAGE of immunoprecipitates using either patient 1 (R16316) or 2 (R11040) crosslinked serum precipitated with unlabelled K562 cell extract. The molecular weight markers are indicated in kDa on the left and the bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated on the right.

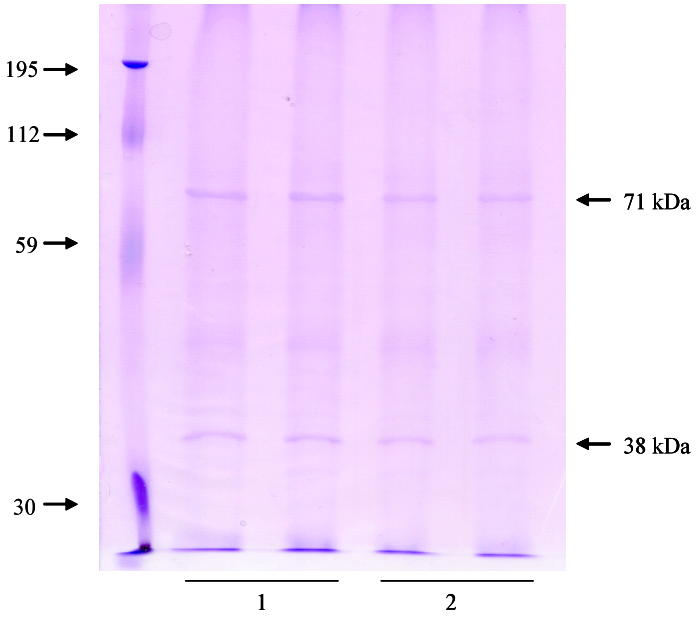
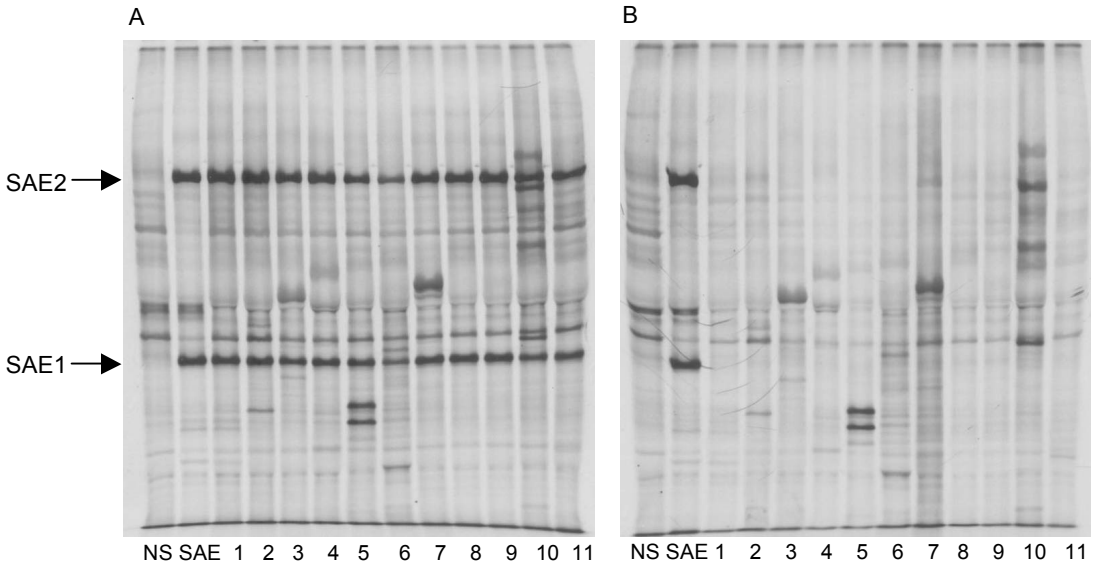


Figure 12: SAE Immunodepletion

Autoradiogram of 10% SDS-PAGE of immunoprecipitates using anti-SAE positive patient serum and either normal serum depleted [^{35}S] methionine – labelled cell extract (A) or SAE depleted [^{35}S] methionine – labelled cell extract (B) NS – IPP of normal serum and undepleted [^{35}S] methionine – labelled cell extract, SAE – IPP using anti-SAE positive serum and undepleted 35S methionine. Bands corresponding to SAE1 (40 kDa band) and SAE2 (90 kDa band) are indicated.



4.3.3 Anti-p155/140: autoantibodies targeting 155/140 kDa doublet protein in adult dermatomyositis

Identification of anti-p155/140 autoantibodies

The RNHRD IIM study described in Chapter 3 identified seven DM patients with the same previously unidentified autoantibody pattern and similar clinical features:

R7505	R13064
R8315	R16815
R9316	R 20125
R11248	

Following IPP testing, sera from the above cases of adult DM recognised two distinct proteins forming a doublet with molecular weights of 155 kDa and 140 kDa (see Figure 13). In each case a weak, non-specific nuclear pattern was detected on IIF (data not shown).

Immunodepletion studies were undertaken in order to ascertain whether the IPP pattern seen in the adult DM cases was due to precipitation of the same autoantigens. Immunoprecipitations on the different anti-p155/140 kDa positive sera were completed using cell extracts depleted with a reference index case anti-p155/140 serum (R7505), or with healthy control serum. All sera were positive for the p155/140 kDa bands using healthy control depleted cell extract but were negative for the p15/140 kDa bands with the index case (R7505) depleted extract (see Figure 14). These results provide confirmation that the serum from the index case and the six additional patients contain the same autoantibody specificity, anti-p155/140. None of the seven anti-p155/140 positive sera were found to co-immunoprecipitate any other recognised MSAs.

Frequency of anti-p155/140 autoantibodies

A total of seven out of 50 (14%) adult IIM patients were positive for autoantibodies to the p155/140 kDa doublet. Anti-p155/140 was only detected in adult DM cases with a frequency of 26.9%. Anti-p155/140 autoantibodies were not found in any of the disease or normal control groups.

Clinical features of anti-p155/140-positive patients

Findings are summarised in Table 11 and Table 12. Four adult DM patients with anti-p155/140 had a history of malignancy, which was diagnosed either at onset or

within three months of their DM. The remaining three anti-p155/140 patients had clinically-amyopathic DM (CADM) based on clinical findings and normal creatinine kinase levels since diagnosis, and no history of malignancy (median seven years from diagnosis to time of study).

In comparison to anti-p155/140 negative adult DM patients, anti-p155/140-positive patients had a higher frequency of cutaneous lesions; including V-sign rash, Shawl sign rash, heliotrope rash and Gottron's lesions. In addition, systemic manifestations including dysphagia were more frequent. No anti-p155/140-positive patients had interstitial pneumonia.

There was a significant association with anti-p155/140-positivity and malignancy (P_{corr}=0.03, odds ratio 7.3). The types of cancer seen in this group of patients were varied but all patients had evidence of solid organ malignancy.

Table 11: Clinical features of anti-p155/140-positive adult DM *vs.* anti-p155/140-negative adult DM ᵀ

	Anti-p155/140 (adult DM)	
	Positive (n=7)	**Negative (n=19)**
Median age at diagnosis (yrs)	54 (33, 71)	51 (49, 58)
Females	71.4	89.5
Heliotrope rash	85.7	63.2
Gottron's papules	100	84.2
Periungal erythema	71.4	63.2
V-sign rash	71.4	26.3
Shawl-sign rash	85.7	26.3
Dysphagia	57.1	21.0
Weight loss	28.6	26.3
Weakness	57.1	73.7
Elevated CK	57.1	78.9
Interstitial pneumonia	0	31.6
CAM	57.1 #	0
Arthritis	28.6	36.8
Calcinosis	14.3	0
Mechanics hands	0	15.8

ᵀValues are in percentages unless otherwise indicated

CK: creatinine kinase

CAM: cancer-associated myositis

#P_{corr}=0.03 compared to anti-p155/140 negative DM patients (P=0.002), odds ratio 7.3, 95% CI 2.56-20.99)

Table 12: Anti-p155/140-positive patients (types of malignancy)

Patient	Diagnosis	Age at onset	Malignancy
1	DM	82	Small cell carcinoma adrenal metastasis
2	DM	71	Bone and liver metastasis
3	DM	50	Breast carcinoma
4	DM	77	Gastric carcinoma
5	CADM	28	No
6	CADM	24	No
7	CADM	56	No

CADM: clinically-amyopathic dermatomyositis

Figure 13: IPP anti-p155/140 autoantibodies

Immunoprecipitation of p155/140 kDa autoantigens and other selected MSAs. 10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extracts. Sera used for immunoprecipitation include Lanes 1-6; adult anti-p155/140 positive serum, Lane 7; threonyl tRNA synthetase, Lane 8; alanyl tRNA synthetase, Lane 9; histidyl tRNA synthetase, Lane 10; Mi-2, Lane 11; signal recognition particle and Lane 12; normal serum. Positions of the p155 and p140 antigens are indicated on the left. Positions of the Mi-2 bands at 240, 65 and 63 kDa are indicated. The 54, 83 and 108 kDa molecular weight markers correspond to signal recognition particle, threonyl tRNA synthetase and alanyl tRNA synthetase respectively.

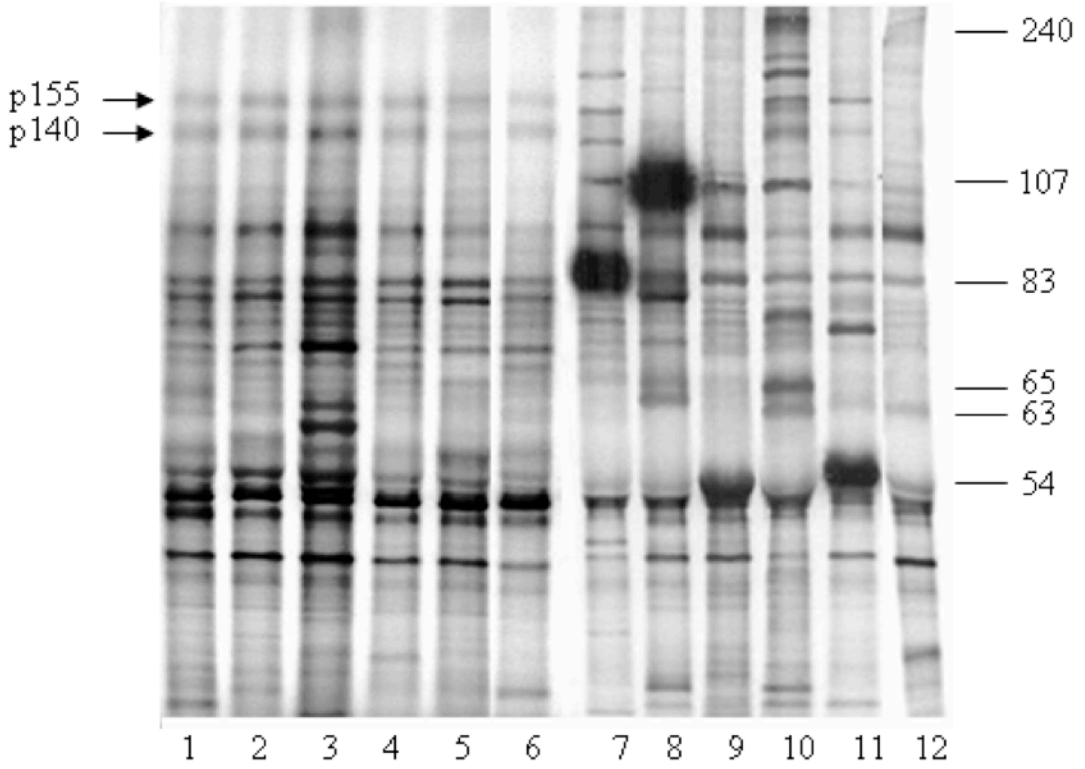
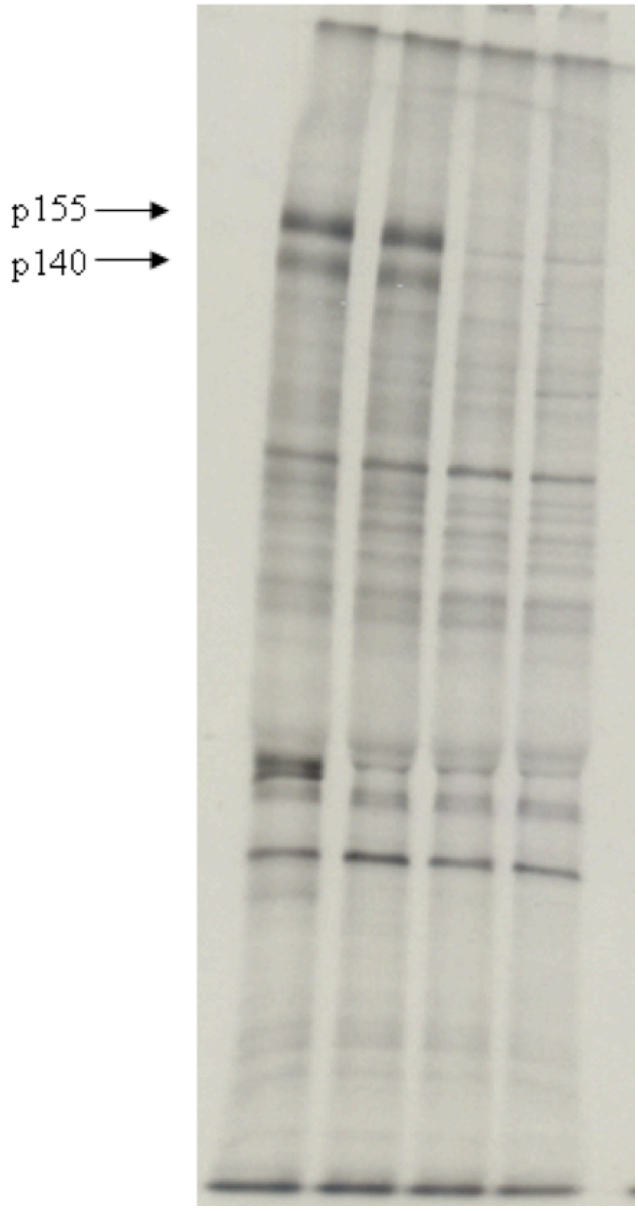


Figure 14: Immunodepletion experiments (anti-p155/140)

Immunodepletion experiments - Autoradiogram of 10% SDS-PAGE of immunoprecipitates using adult positive anti-p155/140 serum: R7505, R8315 and R9316. Immunoprecipitation was performed with control [^{35}S] methionine-labelled cell extract or [^{35}S] methionine-labelled cell extract depleted with either normal serum (NS) or adult positive anti-p155/140 serum (R7505). The bands corresponding to the p155 and p140 autoantigens are indicated.



	1	2	3	4
Depletion serum	None	NS	R7505	R7505
IPP serum	R8315	R9316	R8315	R9316

4.4 Discussion

This chapter outlines work that characterises three new MSAs in adult IIM. The first is a new anti-ARS (-Zo) that targets phenylalanyl tRNA synthetase, which is the eighth autoantigenic ARS to be identified. The second is an autoantibody that targets SAE, an enzyme involved in post-translational modification, a completely novel system in adult DM. Finally, the clinical specificity of anti-p155/140 autoantibodies are described in patients recruited to the RNHRD IIM cohort study.

4.4.1 Anti-Zo; autoantibodies against phenylalanyl tRNA-synthetase

The aminoacyl tRNA-synthetases are a distinct group of enzymes that catalyse the ATP-dependent binding of an amino acid to its cognate tRNA during protein synthesis. ARS are functionally related enzymes; each amino acid has its own corresponding tRNA-synthetase, except glutamic acid and proline, which have both been shown to be catalysed by a single polypeptide encoding the 'gluprolyl' tRNA synthetase (163). Previously, seven anti-ARS have been described (see Chapters 1 and 3*)* (111, 114-119). ARS belong to one of two classes depending on the amino acid they are responsible for. Both classes are multi-domain proteins with a catalytic domain and an anti-codon binding domain (ensures binding of the correct tRNA to the protein). Five ARS; histidyl (Jo-1), threonyl (PL-7), alanyl (PL-12), asparaginyl (KS) and glycyl (EJ) are class II synthetases found free in the cell cytoplasm. They have three highly conserved motifs, are generally dimeric or tetrameric, and attach their amino acid to the 3' hydroxy (OH) of their tRNA. The other two autoantigenic ARS: isoleucyl (OJ) and tyrosyl (Ha) are class I enzymes with two highly conserved motifs, are generally monomeric, and attach the carboxyl of their target amino acid to the 2' OH of adenosine 76 in the tRNA molecule. Anti-OJ autoantibodies are primarily directed against isoleucyl-tRNA syntheatse but can react against multiple syntheases as part of a class I multi-enzyme complex including leucyl and glutaminyl-tRNA (117). Prior to this study; several ARS have not be shown to be autoantigenic (see Table 13). Either these synthetases are not targeted as part of an autoimmune response or autoantibodies directed against them are extremely rare. It is also feasible that it has not been possible to identify autoantibodies to the remaining synthetases using conventional diagnositic techniques.

Phenylalanyl tRNA synthetase is the eighth autoantigen associated with anti-synthetase syndrome to be identified . Similar to the majority of autoantigenic ARS, phenylalanyl is a class II synthetase but it aminoacylates at the 2' OH of the same adenosine, rather than at 3' OH.

Manifestations of anti-synthetase syndromeare described in Chapters 1 and 3. It is noteworthy that the two index cases identified as having anti-Zo autoantibodies had clinical features consistent with this syndrome. In particular, as well as myositis, both patients developed NSIP, the presence of which influenced treatment. As highlighted in Chapters 1 and 3, a major and sometimes predominant manifestation of ani-synthetase syndrome is interstitial pneumonia, which can be the major factor in terms of prognosis (42, 48). The different manifestations are not universal between patients and it is posible that each anti-ARS may define more specific clinical subsets within the syndrome itself. This is perhaps most notable in patients with non-Jo-1-anti-ARS (-KS, -PL-12, -PL-7) with lung disease in the absence of clinically apparent myositis (42, 48, 118, 144, 145). In a study by Yoshifuji H *et al*, the frequency of intestitial lung involvement in their patients with anti-synthetase syndrome was as high as 95%. Interestingly in terms of interstitial pneumonia being the first disease manifestation; anti-ARS autoantibodies were detected in 77% of this subgroup of patients with lung disease preceeding myositis by a median of 11 months. However, in patients who developed myositis first, anti-ARS were only found in 20%. This study also looked at patients with interstitial pneumonia sine myositis; defined by anti-ARS, lung disease, no muscle disease either clinically or biochemically and no cutaneous IIM signs. All patients in this subgroup had non-Jo-1-anti-ARS (48). Similarly, the index anti-Zo-positive cases in this study initially presented with respiratory symptoms; the onset of myositis with other characteristic features occurring later. It is possible that non-Jo-1-anti-ARS may identify patients at one end of the spectrum. The question remains do anti-ARS have a predictive value in the context of idiopathic interstitial pneumonia i.e. do a proportion of these patients actually have a *"forme fruste"* of autoimmune CTD, as suggested in previous studies (48, 150). Detection of anti-ARS autoantibodies in these patients may influence treatment strategies, as highlighted by Yoshifuji H *et al*, who showed that patients with anti-ARS-associated interstitial pneumonia respond significantly better to corticosteroids compared to "seronegative" interstitial pneumonia. However the anti-ARS-positive group were also shown to have a higher rate of relapse with recurrent lung disease (48). This suggests the need to treat patients aggressively with early intervention, with slower tapering of corticosteroids together with additional immunomodulatory therapy. From a clinical perspective, early identification of this subset of patients may influence management. Furthermore, studies described in Section 1.6.4.2 suggest a potential direct role of both candidate autoantigens and specific anti-ARS autoantibodies in disease pathogenesis that may lead to more specific targeted therapies (80, 107, 135, 164).

Table 13: Aminoacyl-tRNA synthetases

Class I tRNA synthetases	Class II tRNA synthetases
Arginyl	Alanyl (PL-12) ᵀ
Cysteinyl	Asparaginyl (KS) ᵀ
Glutamyl	Histidyl (Jo-1) ᵀ
Glutaminyl*	Glycyl (EJ) ᵀ
Isoleucyl (OJ)* ᵀ	Lysyl
Leucyl*	Phenylalanyl (Zo) ᵀ
Methionyl	Prolyl
Tryptophanyl	Seryl
Tyrosyl (Ha) ᵀ	Threonyl (PL-7) ᵀ
Valyl	

ᵀautoantigenic ARS molecules

*autoantibodies to isoleucyl (OJ) also react against other synthetases as part of a class I multi-enzyme complex including leucyl and glutaminyl-tRNA

4.4.2 Anti-SAE autoantibodies

Work described in this chapter has confirmed that SAE is an important autoantigen target in IIM where it is associated with specific clinical and associations. Anti-SAE, like anti-Mi-2, appears to be highly specific for DM. The frequency of anti-SAE in the AOMIC / RNHRD DM cohort was 8%. This is in comparison to Mi-2 which, depending on detection methods, is seen in 10-20% of DM patients (126, 128). Anti-SAE autoantibody positive DM patients have signs of hallmark cutaneous disease whereas interstitial lung involvement appears to be rare, and when present is mild. Systemic features including weight loss and dysphagia are a common manifestation of this subgroup. Furthermore, anti-SAE may be an important prognostic marker in patients who initially present with cutaneous DM. The majority of anti-SAE positive cases presented with skin lesions first and all went onto develop myositis. This finding requires further validation in larger patient groups such as those presenting with clinically-amyopathic DM under the care of dermatologists. The association with cancer and anti-SAE does not appear as strong as the now well recognised association with anti-p155/140 autoantibodies as described previously (143, 157, 158), and also in this chapter. Interestingly, the frequency anti-SAE autoantibodies with cancer-associated myositis were similar to that found for anti-Mi-2 autoantibodies in a recent study (158). In the future, larger studies will facilitate more detailed investigation of the clinical specificity of anti-SAE in comparison to other DM-specific autoantibodies.

Post-translational modifications are essential for full biological activity and allow a rapid cellular mechanism in response to changes in the environment. However, these modifications can also lead to the creation of novel self-antigens and the development of autoimmunity. Examples of this can be seen with glycosylation and hydroxylation modifications in collagen-induced arthritis, citrullination in rheumatoid arthritis and phosphorylation in SLE (165).

Small ubiquitin-like modifiers (SUMO) play a key role in the post-translational modification of specific proteins. The SUMO family consists of four members of which SUMO-1 is the best characterised. Sumoylation leads to the formation of stable conjugates of target proteins including protein kinases and transcription factors (166-169). Studies have implied a role for sumoylation in nucleo-cytoplasmic transport and signal transduction (167, 168). SUMO has been linked to certain inflammatory diseases. Franz *et al* demonstrated high levels of SUMO-1 mRNA expression in rheumatoid synovial tissue compared to controls. The relevance of

this over-expression is unclear but it was suggested that through inhibition of apoptosis, the survival of synovial fibroblasts may be increased (170). In addition, Janka *et al* have reported the presence of autoantibodies against SUMO-1 and SUMO-2 autoantigens in patients with primary biliary cirrhosis (PBC) (171). PBC is associated with other autoimmune conditions and several case reports have described PBC with co-existent myositis (172, 173).

SAE is a heterodimer composed of the subunits SAE1 and SAE2. Desterro *et al* have shown that the two polypeptides SAE1 and SAE2 have apparent molecular weights of 40 and 90 kDa respectively, with predicted molecular masses of 38 kDa and 72 kDa (174). These results are consistent with the data found in this study, where SAE2 was found to migrate at a higher molecular weight than predicted on SDS-PAGE. SAE functions in an ATP-dependant manner and forms a thioester bond between the SAE2 subunit and SUMO, prior to the transfer of SUMO to the E2 conjugating enzyme (174, 175). IIF staining using the SAE subunits have shown that SAE is distributed throughout the nuclei but excluded from the nucleoli (176). These results are consistent with the IIF staining patterns seen with anti-SAE positive sera, where a coarse speckled nucleolar sparing staining pattern was observed. The additional fine cytoplasmic speckle seen in some anti-SAE-positive cases suggests a limited cytosolic pool of the SAE, as previously shown with the sumolyation of RANGAP 1 (177).

Sumoylation is also implicated in the transcriptional repression of histone deacetylase a component of the nucleosome remodelling and deacetylase complex that also includes the myositis specific autoantigen, Mi-2 (178). Therefore, it is possible that the production of autoantibodies to either SAE or Mi-2 in DM maybe the consequence of the same post-translational modification event.

There are other protein targets that are modified by sumoylation (168). Intriguingly these include a member of the GATA family of transcription factors, GATA-2, which is expressed in adult endothelial cells. GATA-2 plays a key role in the transcriptional regulation of endothelial specific genes including endothelin-1 (ET-1), nitric oxide synthetase and Von-Willebrand factor. Both SUMO-1 and 2 covalently modify GATA-2 regulating ET-1 expression by suppressing promoter gene activity (179). This observation is of interest because endothelial cell injury and subsequent microvasculopathy in both muscle and skin lesions is well described in DM (74, 75, 180).

In summary, one can hypothesize that disruption of sumoylation of specific target proteins either via SUMO and / or the enzyme subunits SAE 1 / 2 play a role in the pathogenesis of certain DM clinical phenotypes. The clinical homogeneity of anti-SAE-positive patients, suggests that this is an important finding.

4.4.3 Anti-p155/140 autoantibodies

Over the last two years, several groups have reported an autoantibody termed anti-p155/140 based on the molecular weights of the polypeptide targets (155 and 140 kDa) in adult DM. Targoff *et al* and Kaji *et al* have identified this autoantibody specificity in 21% and 13% of DM patients respectively (143, 157). In both studies anti-p155/140 positive cases had more severe cutaneous involvement including more frequent V-sign and Shawl-sign rash, and very interestingly an association with malignancy. Furthermore, anti-p155/140 autoantibodies appear to be a negative predictor for interstitial lung disease. We have confirmed this observation in our cohort of adult DM patients. Chinoy *et al* investigated this further in larger cohort of patients from the AOMIC registry and found that the risk of malignancy in anti-p155/140 positive cases was significantly higher than anti-p155/140 negative cases (odds ratio of 23.2, 95% confidence interval 6.1-84.5) (158). These studies, and the work described in this chapter, highlight the future potential of routinely testing for anti-p155/140 to aid clinicians in the detection of cancer-associated myositis. The next step is to further validate the clinical utility of anti-p155/140, which can then be translated into routine clinical practice.

In this RNHRD cohort study only four patients out the overall IIM cohort had a history of malignancy and all were anti-p155/140 autoantibody positive. The diagnosis of cancer was either concurrent or within a few months onset of their DM, in concordance with the findings described by Kaji *et al* (157). Kaji *et al* also described two patients who were anti-p155/140-positive without malignancy and two patients who were clinically amyopathic although it is not clear if they were the same patients. Of interest, in this study, the three patients who were anti-p155/140 positive / malignancy negative also had CADM.

Preliminary work has identified the target of the anti-p155 autoantibodies as transcriptional intermediary factor 1-gamma (TIF1-γ) (159). Also known as Ret fused gene 7 (RFG7), this is a member of unique RING finger proteins involved in cellular differentiation (181). The discovery of the p155/140 autoantigen (TIF1-γ) in DM

patients with cancer-associated myositis may increase our understanding of the relationship between autoimmunity and cancer.

4.5 Appendix

I. Acknowledgements (including work done in conjunction with others)

Mrs J Dunphy (JO) and Mrs P Owen (PO) previously performed ANA IIF on sera from IIM cases as part of the RNHRD (Bath Institute for Rheumatic Diseases) Diagnostic Laboratory Service. I would like to thank them for giving me permission to include this data in this study.

I would also like to acknowledge and thank Dr Zoe Betteridge (ZB). The protein isolation, mass spectrometry work and immunodepletion experiments (anti-Zo and anti-SAE studies) were conducted by Dr Zoe Betteridge. I thank her for giving me permission to include her work in this thesis in conjunction with the clinical studies on anti-Zo and anti-SAE autoantibodies.

I would like to thank all the patients who have attended the RNRHD CTD clinic and given their consent to participate in these studies.

CHAPTER FIVE

RESULTS

Serological subsets and clinical associations of myositis-specific and myositis-associated autoantibodies in juvenile dermatomyositis

5.1 Introduction

5.1.1 Epidemiology

The juvenile idiopathic inflammatory myopathies (IIMs) are a group of rare but chronic systemic autoimmune conditions of childhood. Approximately one-fifth of all cases of IIM start in childhood, with an annual incidence of 2.5-5 cases per million population (19). Juvenile dermatomyositis (JDM) is the most common of the idiopathic inflammatory myopathies (IIM) of children, whereas juvenile polymyositis and inclusion body myositis are rarely described. The reported incidence of JDM ranges between 0.8-4.1 per million children per year (18-20). The peak age of onset is 7 years and most studies show that it is just over twice as common in girls than boys, although this ratio may be more comparable in patients under the age of 6 (19, 24). A younger age of onset may confer a worse overall prognosis and 25% cases occur before the age of 4 years (182).

5.1.2 Clinical features

JDM is chronic and potentially debilitating. Despite improvements in multi-disciplinary treatment approaches JDM is still associated with significant morbidity and mortality. Classic JDM rash is similar to adult DM lesions. Heliotrope rash, Gottron's papules over the fingers or extensor aspects of the limbs, and periungal cuticular overgrowth with capillary dilatation are prominent features. Cutaneous lesions are the presenting feature in the majority (over 50% cases), and proximal muscle weakness is the first symptoms in a quarter (183). JDM is a multi-system disease and most children will have other 'systemic' manifestations at onset, including fever, weight loss, anorexia, mouth ulcers, alopecia and irritability (24). In comparison to adult DM, vasculitis is a more prominent feature leading to various clinical signs including subcutaneous oedema and subsequent skin ulceration. Other systemic involvement includes gastrointestinal (GI) vasculitis causing abdominal pain, GI ulceration, bleeding and perforation (24, 183). Dystrophic calcinosis (in the skin, subcutaneous tissue or muscle), acquired lipodystrophy and contractures are also relatively common manifestations in JDM, and are said to be associated with chronic cutaneous inflammation, longer disease duration and delay in diagnosis / treatment onset (184, 185). JDM-scleroderma overlap (JDM-SSc) is well recognised

in JDM children with a history of Raynaud's phenomenon, sclerodactly and other sclerodermatous skin changes. JDM can also overlap with systemic lupus erythematosus (JDM-SLE). In addition, it is well described that a large number of JDM children can also develop overlap inflammatory arthritis (usually in association with JDM-SSc or SLE) (24, 186). In contrast to adult DM, interstitial pneumonia and cancer-associated myositis are very rare in JDM (53, 55-57). Due to the heterogeneity of the condition with multi-organ involvement, clinical outcomes and prognosis are difficult to predict. Certain clinical features, such as skin ulceration, gastro-intestinal involvement (dysphagia and ischaemic ulceration from chronic endarteropathy) or respiratory disease / respiratory muscle weakness (especially leading to aspiration pneumonia) have been proposed as predictors of severe disease course in JDM (21, 25, 57, 187).

5.1.2 Clinical Assessment

There are now standardised validated clinical tools used in the assessment of JDM both in clinical practice and in clinical trials. These include the Childhood Myositis Assessment Scale (CMAS) and the Childhood Health Assessment Questionnaire (CHAQ) (188-191). Additional measures of disease activity include the Physicians' global assessment using a visual analogue scale (VAS) or Likert scale, and a patient / parent global assessment of overall well-being using the same method (VAS) (192). In addition, the Cutaneous Assessment tool (CAT) has been partially validated for the assessment of skin disease in JDM (193, 194).

Standard laboratory markers include muscle enzymes; creatinine kinase (CK), lactate dehydrogenase (LDH), aldolase, and other inflammatory markers including C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). Magnetic resonance imaging (MRI) of proximal muscle groups is now being frequently used for diagnostic purposes as well assessment of muscle disease activity, and in some cases may be preferred to muscle biopsy. T2 fat suppressed images can demonstrate muscle, subcutaneous and cutaneous inflammation and oedema. T1 weighted images are used to establish the degree of fatty infiltration and muscle atrophy, which is representative of damaged muscle (195-197).

Childhood Myositis Assessment Scale (CMAS) *(188, 189)*

Assessment comprises of a series of timed tasks testing muscle strength, endurance and function. See Chapter 5.5: Appendix I.

Childhood Health Assessment Questionnaire (CHAQ) *(190, 191)*

The CHAQ is a functional questionnaire completed by the patient and / or parent to assess activities of daily living. See Chapter 5.5: Appendix I.

Cutaneous Assessment tool (CAT) *(193, 194)*

The CAT is used in both adult and juvenile DM to assess skin disease measuring both activity and damage, using a *priori* weighting scale. See Chapter 5.5: Appendix I.

5.1.3 Aetiology and pathogenesis

The aetiology of JDM remains unclear, however similar to adult IIM, it is well recognised that genetic susceptibility combined with environmental triggers leads to the development of autoimmunity and thus immune dysfunction. The strongest genetic association for autoimmune connective tissue diseases, including JDM is the human leucocyte region (HLA) within the major histocompatibility complex (MHC). The MHC region consists of clustered multiple alleles coding for proteins central to the immune system. The 8.1 ancestral MHC haplotype, (HLA-B*08-DRB1*03-DQA1*05-DQB1*02) is associated with JDM, where the strongest signal appears to come from the HLA class I region (10, 198-200). Recent work demonstrates that the DQA1*0301 allele confers an additional risk for JDM, whereas certain alleles, DQA1*0201, DQA1*0101 and DQA1*0102 appear protective (200). Certain cytokine polymorphisms, including TNFα-0308A and IL-1 receptor antagonist, are also associated with Caucasian JDM with a higher risk of cutaneous ulceration and calcinosis (201, 202).

Environmental factors appear to play a role in the development of JDM. Reports suggest a temporal association to the disease onset. Various infectious agents have been implicated with the onset of JDM, in particular Group A streptococcus and several viruses including coxsackie, enterovirus and parvovirus (203-206). Seasonal birth patterns have been assessed in juvenile-onset IIM, in comparison to patients with adult-onset DM and healthy controls. It was noted that juvenile-onset IIM Hispanic patients had a seasonal birth pattern significantly different to controls. Birth patterns also differed in patients with certain HLA risk factors including

DRB1*0301 and DQA1*0501 alleles. The authors concluded that birth distributions appear to have a stronger seasonality in juvenile IIM, suggesting a role for perinatal or early-life exposures (95).

Similar to adult IIM, both cellular and humoral mechanisms are involved in pathogenesis of JDM, and recent evidence suggests a role of the innate immune system (17, 73, 74, 207-210). Antibody-mediated complement deposition in the vessels plus dendritic cell (DCs), CD4+ T cell and B cell perivascular and perifascicular infiltration and upregulation of MHC class I and class II molecules by myocytes are characteristic. Vasculopathy plays a major pathophysiological role in JDM. Affected muscle is characterised by endothelial dysfunction and capillary dropout. Clinical features in the skin and GI tract suggest similar pathology (73). Hallmark features on muscle biopsy include oedema of the capillary endothelial wall with subsequent occlusive vasculopathy, perivascular and perimysial inflammatory cell infiltrate, muscle fibre changes including perifascicular atrophy. Evidence of muscle degeneration and regeneration is present with neonatal myosin. Recently an international consensus scoring system for the analysis of JDM muscle biopsy specimens has been published (211). Recent work has highlighted the importance of type 1 inteferons (IFN) in JDM disease mechanisms. Type I IFN is believed to be critical in the propagation of autoimmunity, and this mechanism has been implicated in the pathogenesis of JDM as well as adult DM (212, 213). Studies using microarray technology have demonstrated up-regulation of type-1 IFN induced genes in DM (214, 215). DM muscle contains high numbers of gene transcripts associated with type 1 IFN, the source of which are CD4+ plasmacytoid dendritic cells (pDCs) that have chemokine activity. Similar findings were also noted in DM skin. Finally, the potential role of maternal microchimerism in JDM has been highlighted over the last few years. The presence of maternal cells in peripheral blood and diseased muscle of JDM cases may induce autoimmunity i.e. ‘a graft-versus-host disease’ mechanism (216-218).

5.1.4 Autoantibodies in JDM and JDM-overlap syndromes

Until recently, unlike adult IIM, specific autoantibodies in juvenile IIM especially JDM, were detected infrequently despite the majority being ANA positive on IIF. As in adult IIM, autoantibodies are divided into MSAs and MAAs, the latter typically described in JDM-overlap syndromes (JDM-SSc and JDM-SLE).

Unlike adults the frequency of anti-aminoacyl tRNA synthetase (ARS) autoantibodies in juvenile myositis, in particular JDM, is much lower. Rider *et al* used immunodiffusion and immunoprecipitation (IPP) to screen sera from 77 children with myositis and overlap CTD, and anti-ARS were detected in only 2.6% cases (125). Using the same techniques, Feldman *et al* investigated 42 children; thirty five with JDM and 7 with other forms of IIM, and none had anti-ARS autoantibodies (127). The clinical features of anti-ARS-positive JDM cases are similar to their adult counterparts: moderate to severe weakness, arthritis, Raynaud's phenomenon, mechanic's hands, fevers and interstitial lung disease.

Similar to the ARS molecules, SRP is rarely targeted by autoantibodies in juvenile myositis. Based on previous studies the frequency of anti-SRP is 1% (125, 127) Recently, Rouster-Stevens *et al* described three cases of juvenile anti-SRP myositis in their cohort of 123 children and based on their clinical features the patients were comparable to adults with the same autoantibody specificity. In particular; all three children had severe weakness, very high creatinine kinase (CK) levels, were refractory to treatment and had myofibre necrosis on biopsy (219). Of interest, the ethnic origin of these patients was African American and Love *at al* has previously reported a higher frequency of anti-SRP in adult African American cases (6).

The reported frequency of anti-Mi-2 autoantibodies in JDM or JDM overlap is up to 5% (125, 127). Like adult DM, this autoantibody specificity is described in children with hallmark cutaneous DM lesions including Gottron's papules, heliotrope rash, cuticular overgrowth and rashes on the neck and trunk Moreover, patients may have milder muscle involvement, appear to have a monocyclic clinical course and respond well to therapy (125-127).

MAAs: anti-U1-RNP, anti-PM-Scl, anti-U3-RNP, anti-Ku and anti-topoisomerase are typically detected in children with JDM associated with systemic sclerosis features including scleroderma skin changes and Raynaud's. Based on previous data, collectively the overall frequency in JDM / JDM-overlap is between 15-20% (57, 220, 221).

Previously, the overall frequency of defined MSAs and MAAs in children with IIM was between 20-40%, however using full serological testing, specifically IPP this frequency is now much higher following the studies described in the next two

chapters of this thesis, and earlier reports from Wedderburn *et al*, Targoff *et al* and Oddis *et al* (10, 143, 222).

The work described in this chapter is a clinical and serological study of JDM and JDM-overlap children recruited to the JDRR (UK and Ireland). Chapter 6 describes the work on novel autoantibody specificities in JDM.

5.2 Patients and Methods

5.2.1 Patients and sera

Subjects for the study described in this chapter were recruited from:

- The Juvenile Dermatomyositis Registry and Repository (JDRR), UK and Ireland (24).

The JDRR has recruited patients from 10 centres around the UK (for details see Chapter 5, Appendix III) with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis. All JDM cases had probable or definite disease according to *Bohan and Peter* criteria (1, 2). JDM-scleroderma overlap (JDM-SSc) was defined as JDM children with a history of Raynaud's phenomenon, sclerodactly and other sclerodermatous skin changes (two or more of the above features). Using a standardised proforma demographic and clinical data were recorded at diagnosis and prospectively at subsequent visits, on average every 6 months (see Chapter 2, Section 2.1.2 and 2.1.3, and appendix*)*. The clinical information recorded included specific cutaneous manifestations including the presence of Gottron's lesions, skin ulceration, oedema, calcinosis and the distribution of skin rash over the body. Details on muscle involvement included muscle enzymes (CK and LDH) and CMAS at disease baseline. The development of malignancy (within three years of diagnosis) was not specifically recorded on the clinical proforma. However, clinicians were able to enter free text data on clinically significant events during disease course, which was therefore likely to capture this information. Data were stored using anonymous codes onto a central database. Serum samples were taken at the time of diagnosis and stored at -20^{o}C until required.

5.2.2 Serological Methods

ANA-Immunofluorescence (IIF)

IIF was previously performed by standard methods using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).

Protein Radio-immunoprecipitation (IPP)

Sera stored at -20^{o}C were thawed at room temperature. IPP from K562 cell extracts was performed as described in Chapter 2, see Sections 2.3.2 and 2.3.3. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [^{35}S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS-PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

5.2.3 Ethical approval

All patients gave fully informed written / parental consent to participate and provide biological samples according to the Declaration of Helsinki under both national multi-centre and local ethical committee regulations.

5.2.4 Statistical Analysis

Clinical associations were derived from 2×2 contingency tables using the chi-squared test, or two-tailed Fisher's exact test where individual cells valued five or less. *P* values less than 0.05 were considered as significant (uncorrected *P* values presented in view of small numbers in each autoantibody group). SPSS for Windows (version 14) was used to perform statistical analysis.

5.3 Results

Serum samples for serological typing were available from 162 children recruited to the registry. Full clinical data were available on 160 children and 74% were female. The median age of disease onset was 6 years, inter-quartile range (IQR) 3-9 and median age at diagnosis was 7 years (IQR 4, 10). One hundred and twenty seven cases were Caucasian and 35 cases were non-Caucasian.

The median follow-up from disease onset to the time of data analysis for this study was 48 months (IQR 33, 72) for the overall cohort.

One hundred and thirty seven children had JDM. In this study, 21 children were defined as JDM-SSc. Two children were defined as having indeterminate juvenile myositis, not specifically JDM or JDM-SSc.

5.3.1 Serological profiles

Following ANA IIF and IPP, autoantibody specificity was categorised into recognised MSAs, myositis-associated autoantibodies (MAAs), unidentified / novel or negative. The overall frequency of previously defined MSAs and MAAs in this cohort study is shown in Table 14. Table 15 shows a breakdown of serological results and ethnicity (Caucasian and non-Caucasian).

IPP results

Thirteen out of 162 (8%) cases were positive for previously described MSAs: anti-Jo-1 (1.2%) and anti-Mi-2 (6.8%). No cases were positive for anti-SRP autoantibodies. Anti-Mi-2 was found exclusively in JDM patients, with a frequency of 7.9%, and not in any JDM-overlap patients. Anti-Jo-1 was found in JDM-overlap only. The frequency of 'known' MSA-positive in JDM was 8% compared to 9.5% in JDM-overlap.

The overall frequency of MAAs in this study cohort was 14.2%: anti-U1-RNP (5.6%), anti-PM-Scl (6.8%), anti-U3-RNP (0.6%), anti-Ku (0.6%) and anti-Topoisomerase (0.6%). There was a much higher frequency of MAA-positive cases in JDM-overlap (81%) compared to MAA-positive JDM (4.4%). Of note, anti-U1-RNP and anti-PM-Scl was found in higher frequency in Caucasians compared to non-Caucasian children.

Novel autoantibodies were detected in 40% of the overall cohort, and these specificities were found exclusively in JDM cases with a frequency of 47.4% (this work is described in detail in Chapter 6). In the overall cohort, 37.6% of cases were either negative or had undefined bands on IPP.

See Figures 15A-C for examples of MAAs and MSAs in JDM study.

For full IPP results see Chapter 5.5, Appendix II.

ANA IIF results

For overall results see Chapter 5.5, Appendix II.

In total, ANA IIF was performed on 124 samples (there were insufficient sera in 38 samples for both ANA IIF and IPP, therefore only IPP was performed). Out of 124 cases, 96 (77.4%) were ANA positive by IIF.

Figure 15A: Immunoprecipitation of MSAs and MAAs in JDM cohort study

10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extracts.

JDM sera used for immunoprecipitation: Lane 1; anti-Mi-2, Lane 5; anti-Mi-2, Lane 7; anti-Mi-2, Lane 9; anti-U1-RNP.

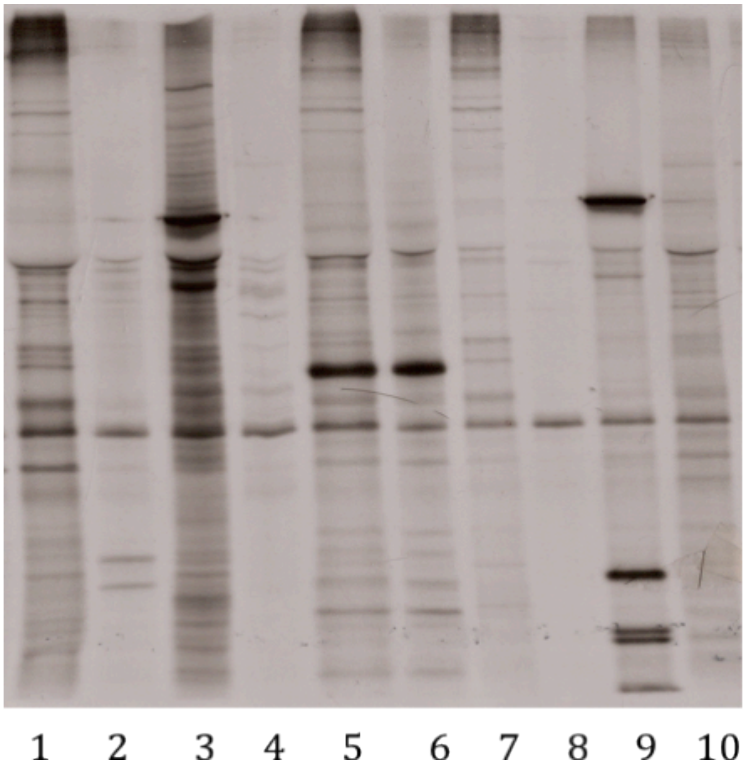


Figure 15B: Immunoprecipitation of MSAs and MAAs in JDM cohort study

10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extracts.

JDM sera used for immunoprecipitation: Lane 1; anti-U1-RNP, Lanes 2 and 9; anti-PM-Scl, Lane 3 and 5; novel anti-p155/140 (see Chapter 6), Lane 4; possible anti-U1-RNP, Lane 6; novel anti-p140 (see Chapter 6).

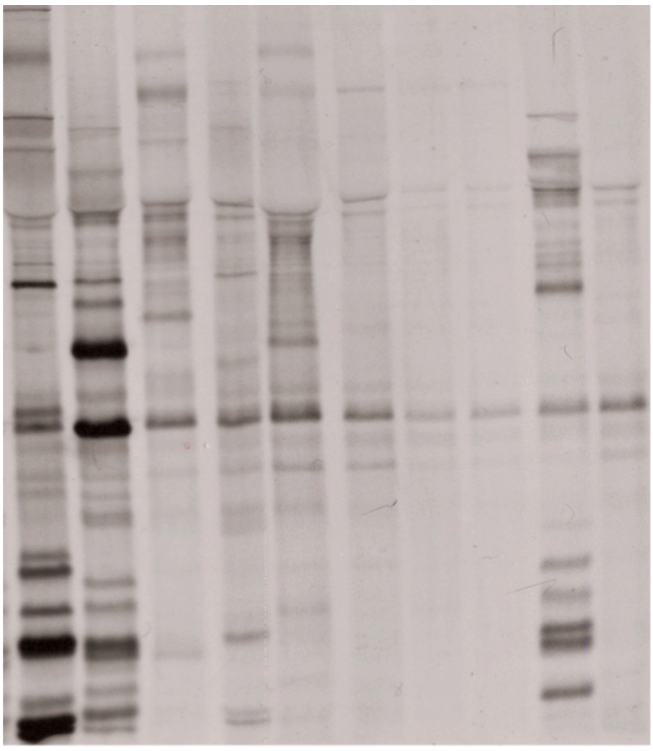


Figure 15C: Immunoprecipitation of MSAs and MAAs in JDM cohort study

10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extracts.

JDM sera used for immunoprecipitation: Lane 1; anti-Ku and anti-U3-RNP, Lane 3; anti-Mi-2, Lane 7; novel anti-p140 (see Chapter 6), Lane 11; anti-U1-RNP.

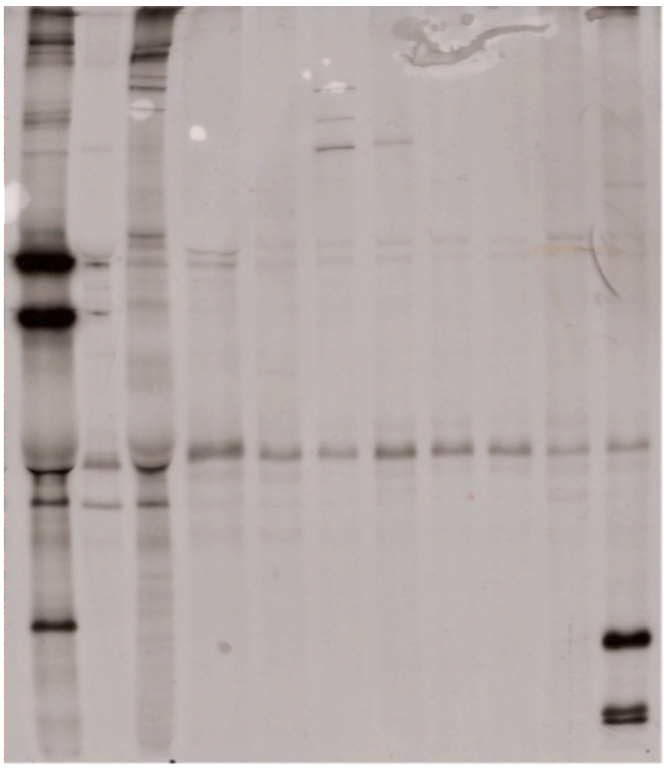


Table 14: Myositis-specific autoantibodies and Myositis-associated autoantibodies in JDM and JDM-overlap cohort

Autoantibody	JDM n=137	JDM-overlap n=21	Other myositis n=2	Overall n=162
MSAs				
Anti-ARS				
Anti-Jo-1	0	9.5	0	1.2*
Anti-PL-12	0	0	0	0
Anti-PL-7	0	0	0	0
Anti-EJ	0	0	0	0
Anti-OJ	0	0	0	0
Anti-KS	0	0	0	0
Anti-Ha	0	0	0	0
Anti-Zo	0	0	0	0
Anti-SRP	0	0	0	0
Anti-Mi-2	7.9	0	0	6.8
MAAs				
Anti-U1-RNP	2.2	28.6	0	5.6
Anti-PM-Scl	2.2	38.1	0	6.8
Anti-U3-RNP	0	4.8	0	0.6
Anti-Ku	0	4.8	0	0.6
Anti-Topo	0	4.8	0	0.6
Novel antibodies See Chapter 6	47.4	0	0	40.1
Unknown bands	16.1	9.5	50	15.4
Negative	25.5	0	50	22.2

Values shown are in percentages

MSAs: myositis-specific autoantibodies

MAAs: myositis-associated autoantibodies

Topo: Topoisomerase (Scl-70)

*One anti-Jo-1-positive case also positive for anti-Ro autoantibodies

Table 15: Myositis-specific autoantibodies and Myositis-associated autoantibodies in JDM and JDM-overlap cohort (ethnic groups)

Autoantibody	JDM Caucasian n=108	JDM Non-Caucasian n=31	JDM-overlap Caucasian n=19	JDM-overlap Non-Caucasian n=4
MSAs				
Anti-ARS (Jo-1)	0	0	10.5	0
Anti-SRP	0	0	0	0
Anti-Mi-2	8	6.5	0	0
MAAs				
Anti-U1-RNP	1.9	0	31.6	25
Anti-PM-Scl	0.8	6.5	42.1	0
Anti-U3-RNP	0	0	5.3	0
Anti-Ku	0	0	0	25
Anti-Topo	0	0	5.3	0
Novel antibodies See Chapter 6	42.6	61.3	0	0
Unknown bands	17.6	9.7	5.3	25
Negative	27.8	16.1	0	0

Values shown are in percentages

MSAs: myositis-specific autoantibodies / MAAs: myositis-associated autoantibodies

Topo: Topoisomerase (Scl-70)

5.3.2 Clinical associations of myositis autoantibodies in study cohort

The overall clinical features of the entire cohort are shown in Table 16. The main clinical features of MSAs (anti-Jo-1 and –Mi-2) and MAAs are shown in Table 17. Two cases were anti-Jo-1-positive with JDM-overlap. The median age at onset was 9 years and both cases were females. Both children had DM rash with myositis, arthritis, sclerodermatous puffy fingers and Raynaud's phenomenon. This MSA subset had no complicating features such as skin ulceration or calcinosis, however the numbers are small. One anti-Jo-1-positive child also had interstitial pneumonia and mechanic's hands.

Anti-Mi-2 was detected in 11 children and all had JDM with hallmark cutaneous changes and a wide distribution of rash: Gottron's papules (100%), cutaneous oedema 63%, periorbital (100%) and periungal overgrowth with capillary changes (91%). The median age at onset was 12.5 years and 90% cases were girls.

MAAs (specifically anti-U1-RNP, -PM-Scl, -U3-RNP, -Ku, -Topo) were detected in 17 children with JDM-overlap and 6 children with a diagnosis of JDM. MAA-positive children had a median age at onset of 9 years, and 78% were female. Sixty-five% of MAA-positive cases had Gottron's papules, with periorbital rash (35%), periungal changes (57%) and rash on the trunk (17%). Other features were frequent: arthritis (65%), scleroderma skin changes (61%), Raynaud's (48%), calcinosis (21%) and lipoatrophy (35%). Both anti-PM-Scl and anti-U1-RNP autoantibodies were present in 5-7% of juvenile IIM respectively (a similar frequency to anti-Mi-2).

When comparing anti-Mi-2-positive children with MAA-positive children the frequency of skin lesions - Gottron's papules (P=0.034) and cutaneous oedema (P=0.021), distribution of rash – periorbital (P<0.001), were significantly higher in the anti-Mi-2 group. Sclerodermatous features were significantly more frequent in the MAA-positive group (P<0.001).

Table 16: Overall clinical features of the UK JDM Cohort study (n=160)

Clinical feature	Frequency (%)
Female	73.8
Caucasian	79.4
Skin disease	
Gottron's	82.9
Ulceration	25.3
Lipoatrophy	14
Oedema	34.8
Calcinosis	23.3
Distribution of rash	
Periorbital	71.4
Periungal	66.2
Trunk	14.3
Small joints	70.8
Large joints	51.3
Other features	
Arthritis	44.7
Sclerodermatous features	12.6
Raynaud's phenomenon	16.6
Dysphagia	31.4
Mouth ulcers	27
Alopecia	29.7
Interstitial pneumonia	5

Values are in percentages

Table 17: Clinical features of MSAs and MAAs in JDM and JDM-overlap cohort

	Anti-Jo-1 n=2 (%)	Anti-Mi-2 n=11 (%)	MAA n=23 (%)
Median age (onset)	9	12.5	7
Median age (diagnosis)	9	12.5	9
Female	100	90	78
Type of skin lesion			
Gottron's papules	100	100 *	65 *
Ulceration	0	9	13
Oedema	0	63 **	17 **
Calcinosis	0	9	21
Lipoatrophy	0	9	35
Mechanic's hands	50	0	0
Rash distribution			
Periorbital	100	100 δ	35 δ
Periungal	100	91	57
Small joints	100	82	65
Large joints	50	45	30
Trunk	0	0	17
Other features			
Arthritis	100	36	65
Scleroderma	100	0 α	61α
Raynaud's	100	9	48
Dysphagia	0	45	30
Mouth ulcers	0	36 αα	4 αα
Alopecia	100	30	26
Interstitial pneumonia	50	0	4
Myositis			
Raised CK	100	70	76

Values are in percentages, unless otherwise stated, (n/a: full data not available)

* $P_{(uncorr)}$=0.034: anti-Mi-2-positive *versus* MAA-positive

** $P_{(uncorr)}$=0.021: anti-Mi-2-positive *versus* MAA-positive

δ P<0.001: anti-Mi-2-positive *versus* MAA -positive

α P<0.001: anti-Mi-2-positive *versus* MAA-positive

αα$P_{(uncorr)}$=0.05: anti-Mi-2-positive *versus* MAA-positive

5.4 Discussion

This chapter describes a serological study of JDM and JDM-overlap in children recruited to the JDRR. The aim of the work in this Chapter was to establish the frequency of known MSAs and MAAs, and describe the clinical associations in this JDM UK cohort.

In comparison to previous serological studies in juvenile IIM summarised in several review articles (221, 223, 224), the overall frequency of MSAs and MAAs are similar, especially the anti-Mi-2 and MAAs subsets. In a recent study by Espada *et al*, 64 Argentine Caucasian children with juvenile IIM (including 40 JDM cases and 17 myositis overlap cases) were screened using IPP and RNA immunoprecipitation. The frequency of anti-Mi-2 was 6%, anti-PM-Scl was 3% and anti-U1-RNP autoantibody was 11%. No children were positive for anti-ARS autoantibodies (225). In this cohort study, the frequency of anti-ARS autoantibodies was generally less than previously described (125, 127), with less anti-Jo-1-positives and no children testing positive for non-Jo-1-ARS. In contrast to adult IIM where autoantibodies to ARS are a common target (as described in Chapter 3), this group of MSAs are detected infrequently in juvenile myositis. Although the numbers of children with anti-ARS autoantibodies were small, the clinical manifestations do resemble that of their adult counterparts with the anti-synthetase syndrome (see Chapter 3) (46). Anti-SRP autoantibodies were not detected in this cohort, which may reflect the ethnic make-up of this cohort (the majority of cases being Caucasian) with no cases of 'juvenile PM' being recruited to the JDRR at the time of this study. Typically anti-SRP-positivity is rare in juvenile IIM anyway and a recent report has highlighted how this MSA may be generally more prevalent in Afro-American children (219). Anti-Mi-2 was detected in JDM children with hallmark cutaneous changes, a wide distribution of rash, and a relatively higher frequency of cutaneous / subcutaneous oedema in comparison to the anti-Jo-1 and MAA subgroups. In contrast, extra-muscular / cutaneous complications were relatively less frequent except for dysphagia. The frequency of anti-Mi-2 in both juvenile and adult myositis is similar.

The frequency of MAAs in this juvenile cohort is similar to that seen in adult IIM, especially when comparing JDM-SSc patients with adult myositis/CTD overlap (139). The most common MAA specificities were anti-U1-RNP and anti-PM-Scl, with small number of children testing positive for anti-U3-RNP, -Topo, and -Ku. There were no patients with other systemic sclerosis-associated autoantibodies e.g. anti-centromere or anti-RNA polymerase I, II or III, which highlights differences between

juvenile and adult myositis-SSc overlap populations (139, 226). Children with SSc, including the diffuse form of the disease, have similar serological profiles. Anti-PM-Scl and anti-U1-RNP autoantibodies are detected in a higher frequency compared to adult onset SSc. Other specificities including anti-centromere, anti-RNA polymerase and anti-topoisomerase are more common in adult SSc (226, 227). In general, MAA-positive children had less classic skin DM lesions but myositis with more arthritis, scleroderma skin changes, Raynaud's and lipoatrophy. In comparison to anti-Jo-1 and anti-Mi-2-positive children, MAA-positives had a relatively higher frequency of calcinosis, and this observation may reflect the predominant overlap features with scleroderma. In general, overlap features are seen in about approximately 10% of juvenile-onset IIM (21, 22). In this study, 12% of children were defined as having JDM-SSc overlap, and although full data was not available to make any conclusions regarding severity of muscle disease, it has been observed that children with overlap may have milder muscle disease with lower disease activity markers (21).

In summary, the overall frequency of recognised MSA or MAAs in this cohort was around 20%. Therefore, most children with myositis are either autoantibody negative or have autoantibodies that target novel autoantigens. The majority have novel specificities and this work is described in Chapter 6.

5.5 Appendix I

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CHILDHOOD MYOSITIS ASSESSMENT SCALE (CMAS) SCORING SHEET

1. **HEAD LIFT:**
   0 = Unable 3 = 30-59
   1 = 1-9 sec 4 = 60-119 sec
   2 = 10-29 5 = ≥ 2 min # of sec ____

2. **LEG RAISE/TOUCH OBJECT:**
   0 = Unable to lift leg off table.
   1 = Able to clear table, but cannot touch object (examiner's hand).
   2 = Able to lift leg high enough to touch object (examiner's hand).

3. **STRAIGHT LEG LIFT/DURATION:**
   0 = Unable 3 = 30-59 sec
   1 = 1-9 sec 4 = 60-119 sec
   2 = 10-29 sec 5 = ≥ 2 min #of sec ____

4. **SUPINE TO PRONE:**
   0 = Unable. Has difficulty even turning onto side; able to pull right arm under torso only slightly or not at all.
   1 = Turns onto side fairly easily, but cannot fully free right arm and is unable to fully assume a prone position.
   2 = Easily turns onto side; has some difficulty freeing arm, but fully frees arm and fully assumes a prone position.
   3 = Easily turns over, fully frees right arm with no difficulty.

5. **SIT-UPS :**
   Hands on thighs, with counterbalance ____
   Hands across chest, with counterbalance ____
   Hands behind head, with counterbalance ____
   Hands on thighs, without counterbalance ____
   Hands across chest, without counterbalance ____
   Hands behind head, without counterbalance ____
   Total Sit-up Score (0-6) ____

6. **SUPINE TO SIT:**
   0 = Unable by self.
   1 = Much difficulty. Very slow, struggles greatly, barely makes it. Almost unable.
   2 = Some difficulty. Able, but is somewhat slow, struggles some.
   3 = No difficulty.

7. **ARM RAISE/STRAIGHTEN:**
   0 = Cannot raise wrists up to the level of the A-C joint.
   1 = Can raise wrists at least up to the level of the A-C joint, but not above top of head.
   2 = Can raise wrists above top of head, but cannot raise arms straight above head so that elbows are in full extension.
   3 = Can raise arms straight above head so that elbows are in full extension.

8. **ARM RAISE/DURATION:** Can maintain wrists above top of head for:
   0 = Unable 3 = 30-59 sec
   1 = 1-9 sec 4 = ≥ 60 sec
   2 = 10-29 sec #of sec _____

9. **FLOOR SIT:** Going from a standing position to a sitting position on the floor:
   0 = Unable. Afraid to even try, even if allowed to use a chair for support. Child fears that he/she will collapse, fall into a sit, or harm self.
   1 = Much difficulty. Able, but needs to hold onto a chair for support during descent. Unable, or unwilling to try if not allowed to use a chair for support.
   2 = Some difficulty. Can go from stand to sit without using a chair for support, but has at least some difficulty during descent. May need Gower's. Descends somewhat slowly and/or apprehensively; may not have full control or balance as maneuvers into a sit.
   3 = No difficulty. Requires no compensatory maneuvering.

10. **ALL FOURS MANEUVER:**
    0 = Unable to go from a prone to an all-fours position.
    1 = Barely able to assume and maintain an all-fours position. Unable to raise head to look straight ahead.
    2 = Can maintain all-fours position with back straight and head raised (so as to look straight ahead). But, cannot creep (crawl) forward.
    3 = Can maintain all-fours, look straight ahead and creep (crawl) forward.
    4 = Maintains balance while lifting and extending one leg.

11. **FLOOR RISE:** Going from a kneeling position on the floor to a standing position:
    0 = Unable, even if allowed to use a chair for support.
    1 = Much difficulty. Able, but needs to use a chair for support. (Unable if not allowed to use a chair.)
    2 = Moderate difficulty. Able to get up without using a chair for support, but needs to place one or both hands on thighs/knees or floor. (Unable without using hands.)
    3 = Mild difficulty. Does not need to place hands on knees, thighs or floor, but has at least some difficulty during ascent.
    4 = No difficulty.

12. **CHAIR RISE:**
    0 = Unable to rise up from chair, even if allowed to place hands on sides of chair seat.
    1 = Much difficulty. Able, but needs to place hands on sides of seat. Unable if not allowed to place hands on sides of seat.
    2 = Moderate difficulty. Able, but needs to place hands on knees/thighs. Does not need to place hands on sides of seat.
    3 = Mild difficulty. Does not need to place hands on seat, knees or thighs but has at least some difficulty during ascent.
    4 = No difficulty.

13. **STOOL STEP:**
    0 = Unable.
    1 = Much difficulty. Able, but needs to place one hand on exam table (or examiner's hand).
    2 = Some difficulty. Able, does not need to use exam table for support, but needs to use hand on knee/thigh.
    3 = Able. Does not need to use exam table or hand on knee/thigh.

14. **PICK-UP:**
    0 = Unable to bend over and pick up pencil off floor.
    1 = Much difficulty. Able, but relies heavily on support gained by placing hands on knees/thighs.
    2 = Some difficulty. Has some difficulty (but not "much-difficulty"). Needs to at least minimally and briefly place hand(s) on knees/thighs for support. Is somewhat slow.
    3 = No difficulty. No compensatory maneuver necessary.

The maximum possible total score for the 14 maneuvers is 52 (52 "points of muscle strength/function").

PATIENT_____ **DATE**_____

TOTAL CMAS SCORE: _____

5.5 Appendix I

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CHILDHOOD HEALTH ASSESSMENT QUESTIONNAIRE

In this section we are interested in learning how your child's illness affects his/her ability to function in daily life. Please feel free to add any comments on the back of this page. In the following questions, please check the one response which best describes your child's usual activities (averaged over an entire day) OVER THE PAST WEEK. **ONLY NOTE THOSE DIFFICULTIES OR LIMITATIONS WHICH ARE DUE TO ILLNESS**. If most children at your child's age are not expected to do a certain activity, please mark it as "Not Applicable". **For example, if your child has difficulty in doing a certain activity or is unable to do it because he/she is too young but not because he/she is RESTRICTED BY ILLNESS, please mark it as "NOT Applicable".**

	Without ANY Difficulty	With SOME Difficulty	With MUCH Difficulty	UNABLE To do	Not Applicable
DRESSING & GROOMING					
Is your child able to:					
- Dress, including tying shoelaces and doing buttons?	☐	☐	☐	☐	☐
- Shampoo his/her hair?	☐	☐	☐	☐	☐
- Remove socks?	☐	☐	☐	☐	☐
- Cut fingernails?	☐	☐	☐	☐	☐
ARISING					
Is your child able to:					
- Stand up from a low chair or floor?	☐	☐	☐	☐	☐
- Get in and out of bed or stand up in a crib?	☐	☐	☐	☐	☐
EATING					
Is your child able to:					
- Cut his/her own meat?	☐	☐	☐	☐	☐
- Lift up a cup or glass to mouth?	☐	☐	☐	☐	☐
- Open a new cereal box?	☐	☐	☐	☐	☐
WALKING					
Is your child able to:					
- Walk outdoors on flat ground?	☐	☐	☐	☐	☐
- Climb up five steps?	☐	☐	☐	☐	☐

*** Please check any AIDS or DEVICES that your child usually uses for any of the above activities:**

- Cane	☐	- Devices used for dressing (button hook, zipper pull, long-handled shoe horn, etc.)	☐
- Walker	☐	- Built up pencil or special utensils	☐
- Crutches	☐	- Special or built up chair	☐
- Wheelchair	☐	- Other (Specify:_____)	☐

*** Please check any categories for which your child usually needs help from another person BECAUSE OF ILLNESS:**

- Dressing and Grooming	☐	- Eating	☐
- Arising	☐	- Walking	☐

	Without ANY Difficulty	With SOME Difficulty	With MUCH Difficulty	UNABLE To do	Not Applicable
HYGIENE					
Is your child able to:					
- Wash and dry entire body?	☐	☐	☐	☐	☐
- Take a tub bath (get in and out of tub)?	☐	☐	☐	☐	☐
- Get on and off the toilet or potty chair?	☐	☐	☐	☐	☐
- Brush teeth?	☐	☐	☐	☐	☐
- Comb/brush hair?	☐	☐	☐	☐	☐
REACH					
Is your child able to:					
- Reach and get down a heavy object such as a large game or books from just above his/her head?	☐	☐	☐	☐	☐
- Bend down to pick up clothing or a piece of paper from the floor?	☐	☐	☐	☐	☐
- Pull on a sweater over his/her head?	☐	☐	☐	☐	☐
- Turn neck to look back over shoulder?	☐	☐	☐	☐	☐
GRIP					
Is your child able to:					
- Write or scribble with pen or pencil?	☐	☐	☐	☐	☐
- Open car doors?	☐	☐	☐	☐	☐
- Open jars which have been previously opened?	☐	☐	☐	☐	☐
- Turn faucets on and off?	☐	☐	☐	☐	☐
- Push open a door when he/she has to turn a door knob?	☐	☐	☐	☐	☐
ACTIVITIES					
Is your child able to:					
- Run errands and shop?	☐	☐	☐	☐	☐
- Get in and out of a car or toy car or school bus?	☐	☐	☐	☐	☐
- Ride bike or tricycle?	☐	☐	☐	☐	☐
- Do household chores (e.g. wash dishes, take out trash, vacuuming, yardwork, make bed, clean room)?	☐	☐	☐	☐	☐
- Run and play?	☐	☐	☐	☐	☐

*** Please check any AIDS or DEVICES that your child usually uses for any of the above activities:**

- Raised toilet seat	☐	- Bathtub bar	☐
- Bathtub seat	☐	- Long-handled appliances for reach	☐
- Jar opener (for jars previously opened)	☐	- Long-handled appliances in bathroom	☐

*** Please check any categories for which your child usually needs help from another person BECAUSE OF ILLNESS:**

- Hygiene	☐	- Gripping and opening things	☐
- Reach	☐	- Errands and chores	☐

PAIN: We are also interested in learning whether or not your child has been affected by pain because of his or her illness. How much pain do you think your child has had because of his/her illness IN THE PAST WEEK? Place a mark on the line below, to indicate the severity of the pain

No pain 0 ——————————— 100 Very severe pain

GLOBAL EVALUATION: Considering all the ways that arthritis affects your child, rate how he/she is doing by placing a single mark on the line below.

Very well 0 ——————————— 100 Very poor

5.5 Appendix I

Cutaneous Assessment Tool

Full proforma available on:

http://dir-apps.niehs.nih.gov/imacs/index.cfm?action1/4home.main

5.5 Appendix II – Subjects, ANA IIF and IPP results

Study ID	Diagnosis	ANA IIF result	IIP result
UK01PB0001	JDM	Negative	140 kDa band *
UK01SS0002	JDM	Negative	Negative
UK01JR0003	JDM	Weak Homogeneous	155/140 kDa bands *
UK01JA0004	JDM	Weak FSNS	140 kDa band *
UK01ZE0005	JDM overlap	Homogeneous	Ku
UK01MB0006	JDM	FSNS	155/140 kDa bands *
UK01SP0007	JDM	Weak FSNS	155/140 kDa bands *
UK01ZB0008	JDM	Nucleolar	PM-Scl
UK01MV0010	JDM overlap	Weak Homogeneous with Nucleolar	Topoisomerase
UK01LP0016	JDM	Weak FSNS	Negative
UK01AP0017	JDM	Negative	140 kDa band *
UK01JC0018	JDM	Weak FSNS	155/140 kDa bands *
UK01SO0019	JDM	Negative	Negative
UK01SM0020	JDM overlap	Nucleolar	PM-Scl
UK01NT0021	JDM	Negative	Negative
UK01RK0022	JDM overlap	DCNS with Cytoplasmic Speckle	Jo-1
UK01RC0023	JDM	Negative	140 kDa band *
UK01KS0024	JDM	FSNS	Mi-2
UK01SP0025	JDM	Weak FSNS	155/140 kDa bands *
UK01CM0026	JDM	Negative	Unknown bands
UK01IP0027	JDM	DCNS	Unknown bands
UK01AP0028	JDM	Negative	Negative
UK01ES0029	JDM	Weak FSNS	140 kDa band *
UK01ZM0030	JDM overlap	Nucleolar	PM-Scl
UK01JB0031	JDM	Weak FSNS	Unknown bands
UK01JB0032	JDM	Negative	140 kDa band *
UK01FS0033	JDM	Weak FSNS	155/140 kDa bands *
UK01LT0037	JDM	Weak DCNS	Unknown bands
UK01RW0038	JDM	Negative	Unknown bands
UK01LS0039	JDM	DCNS	Unknown bands
UK01AL0040	JDM overlap	Nucleolar	PM-Scl
UK01EM0042	JDM	DCNS	Unknown bands

UK01KH0043	JDM	Homogeneous	Mi-2
UK01CB0047	JDM	Negative	Negative
UK01FU0048	JDM	Homogeneous	Mi-2
UK01GG0049	JDM	Negative	140 kDa band *
UK01JW0050	JDM overlap	Nucleolar with Coarse Speckle	PM-Scl
UK01LW0051	JDM overlap	Nucleolar with Cytoplasmic Speckle	PM-Scl
UK01LP0052	JDM	Negative	Negative
UK01EB0053	JDM	FSNS with Cytoplasmic Speckle	155/140 kDa bands *
UK01RS0054	JDM	FSNS	155/140 kDa bands *
UK01ST0056	JDM	Weak Fine Cytoplasmic Speckle	Negative
UK01EB0057	JDM	FSNS	155/140 kDa bands *
UK01VA0058	JDM	Homogeneous	155/140 kDa bands *
UK01JG0059	JDM	DCNS	Unknown bands
UK01JR0060	JDM	Weak FSNS	155/140 kDa bands *
UK01EK0063	JDM	Weak FSNS	Negative
UK01LC0065	JDM overlap	Nucleolar with Weak Homogeneous	U3-RNP
UK02NS0066	JDM	FSNS	140 kDa band *
UK01KS0068	JDM	Negative	140 kDa band *
UK01JJ0069	JDM	Weak FSNS	155/140 kDa bands *
UK01PB0070	JDM	Negative	140 kDa band *
UK01SB0071	JDM	Weak FSNS	140 kDa band *
UK01BH0073	JDM	Weak FSNS	Unknown bands
UK01CW0074	JDM	FSNS	Mi-2
UK01NW0075	JDM	FSNS	Unknown bands
UK01EB0076	JDM	FSNS	155/140 kDa bands *
UK01JT0077	JDM	Homogeneous	Mi-2
UK08KW0079	JDM	Weak FSNS	Unknown bands
UK04LM0082	JDM overlap	DCNS	U1-RNP
UK04JC0084	JDM	Weak DCNS	Negative
UK04BF0085	JDM overlap	DCNS	U1-RNP
UK04VL0086	JDM overlap	DCNS	U1-RNP
UK10CS0091	JDM	Negative	140 kDa band *
UK05AM0093	JDM	Negative	140 kDa band *
UK04NC0094	JDM	DCNS	Unknown bands

UK04GF0095	JDM overlap	Nucleolar with Discrete Speckle	PM-Scl
UK10KM0096	JDM	Homogeneous	Mi-2
UK09TH0098	JDM	Weak DCNS	140 kDa band *
UK09LW0099	JDM	FSNS	155/140 kDa bands *
UK09CD0101	JDM	DCNS	Negative
UK09SA0104	JDM	Weak Homogeneous	140 kDa band *
UK09HB0105	JDM	Negative	140 kDa band *
UK09LM0110	JDM	FSNS	155/140 kDa bands *
UK06RW0115	JDM	Weak Homogeneous	155/140 kDa bands *
UK01LT0118	JDM	γ	Unknown bands
UK02HC0119	JDM	FSNS	155/140 kDa bands *
UK01EB0120	JDM	Weak Homogeneous	140 kDa band *
UK10AW0122	JDM	DCNS	U1-RNP
UK01KS0125	JDM overlap	Nucleolar with Cytoplasmic Speckle	Jo-1
UK07AO0126	JDM	Weak DCNS	155/140 kDa bands *
UK07LF0128	JDM	γ	Negative
UK07ZM0129	JDM	Weak FSNS	140 kDa band *
UK07MG0130	JDM	FSNS	155/140 kDa bands *
UK07SL0131	JDM	DCNS	140 kDa band *
UK07MP0132	JDM	Weak Cytoskeletal	Unknown bands
UK08IC0135	JDM	Negative	140 kDa band *
UK08CT0136	JDM	FSNS	Unknown bands
UK08KP0137	JDM	Weak Cytoskeletal	Negative
UK08AD0138	JDM	Negative	Negative
UK10SW0139	JDM	DCNS	140 kDa band *
UK01LT0140	JDM	Homogeneous	Mi-2
UK01SD0141	JDM	FSNS	Unknown bands
UK01ET0142	JDM	DCNS	155/140 kDa bands *
UK01NZ0144	JDM	DCNS	Mi-2
UK07LH0148	JDM	Weak FSNS	140 kDa band *
UK08PS0149	JDM	Homogeneous	Mi-2
UK10NE0152	JDM	Negative	140 kDa band *
UK07AH0154	JDM	Weak Cytoplasmic Speckle	Negative
UK10LS0156	JDM	Weak FSNS	Negative
UK01BT0157	JDM	FSNS	155/140 kDa bands *

UK01KN0158	JDM	Weak FSNS	155/140 kDa bands *
UK01LG0161	JDM	FSNS	155/140 kDa bands *
UK01AP0162	JDM	Negative	Negative
UK01CH0164	JDM	Weak FSNS	155/140 kDa bands *
UK01AW0166	JDM	Weak FSNS	140 kDa band *
UK01AW0167	JDM	Negative	Negative
UK01LR0169	JDM	Negative	140 kDa band *
UK01AV0171	JDM overlap	DCNS	U1-RNP
UK01SB0172	JDM	Negative	140 kDa band *
UK09HB0174	JDM	DCNS	U1-RNP
UK01EC0175	JDM	γ	140 kDa band *
UK01IA0176	JDM	Weak Cytoplasmic Speckle	140 kDa band *
UK01JA0177	JDM overlap	DCNS	U1-RNP
UK01JS0178	JDM	Negative	140 kDa band *
UK04CJ0179	JDM	γ	140 kDa band *
UK01TB0180	JDM	γ	Negative
UK01PO182	JDM	DCNS	U1-RNP
UK01LF0183	JDM	Weak Homogeneous with	Negative
UK01SA0185	JDM	Nucleolar	PM-Scl
UK01NN0186	JDM	DCNS	Unknown bands
UK01CH0188	JDM	Weak FSNS	Unknown bands
UK01LF0189	JDM	Homogeneous	Mi-2
UK01GW0190	JDM	Negative	Unknown bands
UK01CL0192	JDM	Negative	Negative
UK07LG0194	JDM	γ	155/140 kDa bands *
UK01EW0196	JDM	Weak Cytoskeletal	Negative
UK01ST0197	JDM	γ	Negative
UK04JN0198	JDM	γ	155/140 kDa bands *
UK07RG0199	JDM	γ	140 kDa band *
UK10CK0200	JDM	γ	Negative
UK01AA0201	JDM	γ	140 kDa band *
UK04HS0203	JDM overlap	Nucleolar	PM-Scl
UK01AD0204	JDM	γ	Negative
UK01CA0206	JDM	γ	Negative
UK10CK0208	JDM	γ	Unknown bands
UK08LG0209	JDM	γ	140 kDa band *

UK01JH0212	JDM	γ	Negative
UK01TM0213	JDM	γ	Negative
UK01RH0217	JDM overlap	DCNS	U1-RNP
UK01AL0220	JDM	γ	155/140 kDa bands *
UK01BP0221	JDM	γ	Unknown bands
UK01TA0222	JDM	γ	140 kDa band *
UK09NR0223	JDM	γ	155/140 kDa bands *
UK01AS0224	JDM	γ	140 kDa band *
UK01RW0225	JDM	γ	140 kDa band *
UK01LB0226	JDM	γ	Negative
UK09AM0227	JDM overlap	γ	Unknown bands
UK01KS0228	JDM	γ	140 kDa band *
UK01GM0229	JDM overlap	Nucleolar	PM-Scl
UK01EF0231	JDM	γ	Negative
UK01AJ0233	JDM	γ	Negative
UK01OW0234	JDM	γ	Mi-2
UK01CW0236	JDM overlap	γ	Unknown bands
UK01SF0237	JDM	γ	Negative
UK01PB0239	Indeterminate	γ	Negative
UK10YK0244	JDM	γ	Negative
UK01RM0246	JDM	γ	Negative
UK01RG0247	JDM	γ	Negative
UK10KS0248	JDM	γ	PM-Scl
UK04CS0250	JDM	γ	Unknown bands
UK01KQ0251	Indeterminate	γ	Unknown bands

FSNS – fine speckle nucleolar sparing

DCNS – diifuse coarse nucleolar sparing

γ - ANA IIF not performed (insufficient serum available)

5.5 Appendix III

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CHAPTER SIX

RESULTS

Identification and characterisation of novel autoantigen systems in juvenile dermatomyositis

6.1 Introduction

Classifying patients using a clinico-serological approach may lead to the identification of more homogeneous subsets within the JDM spectrum and therefore have prognostic implications.

The work described in Chapter 5 demonstrates that around 20% of children with JDM or JDM-overlap have well-defined autoantibody specificities (anti-ARS, anti-Mi-2 and MAAs). Therefore, approximately 80% of children would normally be serologically classified as either being non-specifically ANA positive or in some cases seronegative, when tested by ANA IIF or standard ENA ELISA. Over the last few years, a number of novel autoantibodies have been described in adult IIM (as outlined in Chapters 3 and 4). Similarly, there has been a preliminary report from Oddis *et al* of a new autoantibody termed anti-MJ, which targets a ~140-142 kDa protein in JDM (222). A further autoantigen target with a molecular weight of 155 kDa (reported in most cases as a doublet protein with a second weaker band at 140 kDa) has been described in JDM in a study by Targoff *et al* (143). The same novel specificity anti-p155/140 is detected in adult DM, which was described in the same study (143) and was also seen in the RNHRD adult IIM study (see Chapter 4). Two further studies have described anti-p155/140 in separate adult DM cohorts (157, 158). As previously discussed in Chapter 4, anti-p155/140 in adult DM is associated with more severe skin disease but more significantly has a strong association with malignancy. In the Targoff *et al* study, anti-p155/140 was seen in approximately 30% of their JDM population, however the clinical specificity of this cohort was not described (143).

The purpose of the work described in this chapter was to establish the frequency and to define the clinical significance of anti-p155/140 and anti-p140 autoantibodies in children recruited to the UK JDM Cohort Study. The secondary aims were to confirm whether the same p155/140 autoantigen is targeted in adult DM and to demonstrate p155/140 and p140 are different targets. As presented in Chapter 5, 40% of patients tested by IPP were found to be positive for these two different

autoantibodies. Both autoantibodies appear to define distinct clinical phenotypes within the JDM spectrum. This work has been published in the following journals:

- Gunawardena H, Wedderburn LR, North J, Betteridge Z, Dunphy J, Chinoy H, Davidson JE, Cooper RG, McHugh NJ; Juvenile Dermatomyositis Research Group UK. Clinical associations of autoantibodies to a p155/140 kDa doublet protein in juvenile dermatomyositis. Rheumatology. 2007;47:324-8

- Gunawardena H, Wedderburn LR, Chinoy H, Betteridge ZE, North J, Ollier WE, Cooper RG, Oddis CV, Ramanan AV, Davidson JE, McHugh NJ; Juvenile Dermatomyositis Research Group, UK and Ireland. Autoantibodies to a 140-kd protein in juvenile dermatomyositis are associated with calcinosis. Arthritis Rheum. 2009;60:1807-14.

6.2 Patients and Methods

6.2.1 Patients and sera

Subjects for the study described in this chapter were recruited from:

- The Juvenile Dermatomyositis Registry and Repository, UK and Ireland (24).

Full details are given in Chapter 5 (see Section 5.2.1*).* In brief, the JDRR has recruited patients from 10 centres around the UK (for details see appendix (24)) with juvenile-onset myositis, below the age of 16 years at disease onset and diagnosis, to form the JDM UK Cohort Study. All JDM cases had probable or definite disease according to *Bohan and Peter* criteria (1, 2). Demographic and clinical data using a standardised proforma were recorded at diagnosis and prospectively at subsequent visits, on average every 6 months (see Chapter 2, Section 2.1.2 and 2.1.3, and Appendix*)*. Data were stored using anonymous codes onto a central database. Serum samples were taken at the time of diagnosis and stored at -20^{o}C until required.

6.2.1.1 Study 1 (Anti-p155/140 study)

The first 116 patients recruited to the JDM National Registry and Repository (UK and Ireland) (now termed the JDM UK Cohort Study) were initially studied. The median age at symptom onset was 6 years (IQR 3, 9) and at diagnosis was 7 years (IQR 4, 10). The median follow-up from disease onset to time of this study was 69.5 months (IQR 47.5, 105.2) and 44.2 months (IQR 35.7, 65.8) from date of entry into

the registry to time of this study. Clinical information included the degree of skin involvement; muscle strength tested by the Childhood Myositis Assessment Scale (CMAS) (see Chapter 5, Appendix) (188), Physician's 10-point global assessment (PGA) (visual analogue scale) and serum muscle enzymes. When evaluating muscle involvement, only those JDM patients with documented muscle enzymes at disease onset / diagnosis (with subsequent maximum CK or LDH during follow up) were included for analysis. Similarly, those with a baseline CMAS or PGA at the time of entry to the registry (plus subsequent serial measures) were included. The type of skin lesion, in particular skin ulceration, oedema and Gottron's lesions combined with the distribution (extent) of skin involvement plus CMAS/ PGA score was defined as a marker of disease severity for the purpose of this study.

6.2.1.2 Study 2 (Anti-p140 study)

Patients

Serum samples for serological typing were available from the overall cohort of 162 children recruited to the registry (including the first 116 children initially recruited / analysed in the anti-p155/140 study). Clinical data were available on 160 children and 74% were female. The median age of disease onset was 6 years, inter-quartile range (IQR) 3-9 and median age at diagnosis was 7 years (IQR 4, 10). The median follow-up from disease onset to the time of data analysis for this study was 48 months (IQR 33, 72) for the overall cohort. One hundred and thirty seven children had JDM. JDM-scleroderma overlap (JDM-SSc) is well recognised in JDM children with a history of Raynaud's phenomenon, sclerodactly and other sclerodermatous skin changes. In this study, 21 children were defined as JDM-SSc with two or more of the above features. Two children were defined as having other forms of juvenile myositis, not specifically JDM or JDM-SSc.

Controls

Sera from 124 juvenile disease controls; twenty scleroderma / linear scleroderma, 8 systemic lupus erythematosus and 96 juvenile idiopathic arthritis cases were also analysed. No sera from healthy children were available, reflecting ethical difficulties in studies of this nature. Therefore, sera from 50 healthy adult controls were also serotyped.

6.2.2 Serological methods

Study 1 and 2

Indirect immunofluorescence (IIF)

IIF was performed by standard methods using HEp-2 cells and fluorescein-labelled anti-human IgG immunoglobulin (Sigma, UK).

Protein Radio-Immunoprecipitation (IPP)

IPP from K562 cell extracts was performed as previously described in detail in Chapter 2. Briefly, 10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) at room temperature for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [^{35}S] methionine labelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 50 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS PAGE, enhanced, fixed and dried. Labelled proteins were analysed by autoradiography.

Immunodepletion experiments

Study 1

Immunodepletion experiments were performed using reference anti-p155/140-JDM-positive sera and reference anti-p155/140-adult-positive sera (adult sera from the RHHRD serology study – see Chapter 4, Section 4.3.4), to ascertain if the IPP pattern seen was due to precipitation of the same p155/140 autoantigen. Cell extracts were depleted of autoantibody targets using anti-p155/140-JDM-positive serum or anti-p155/140-adult DM-positive serum and normal serum as a negative control. These extracts were then used in further immunoprecipitations using both juvenile and adult anti-p155/140 positive serum.

As described in Chapter 2, Section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl reference anti-p155/140 serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl [^{35}S] methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using JDM or adult DM serum and either 150µl control [^{35}S]-methionine-

labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using [^{35}S] methionine.

Study 2

Immunodepletion experiments with anti-p140-JDM and anti-p155/140-JDM sera

Immunodepletion experiments were performed using reference anti-140-JDM-positive sera and reference anti-p155/140-JDM-positive sera to confirm that these autoantibodies target different autoantigens. Cell extracts were depleted of autoantibody targets using anti-140-JDM positive serum or anti-p155/140-JDM positive serum and normal serum as a negative control. These extracts were then used in further immunoprecipitations using different JDM anti-p140 and anti-p155/140 positive serum. As described in Chapter 2, Section 2.3.4: duplicate samples each containing 10 mg protein A Sepharose beads in 1 ml IPP buffer and 50 µl reference anti-p140 or reference anti-p155/140 serum were mixed with end-over-end rotation at room temperature for 30 min. Beads were washed four times in 1 ml IPP buffer and 1 tube (A) was placed on ice whilst 150 µl [^{35}S] methionine-labelled K562 cell extract and 350 µl IPP buffer was added to the remaining tube (B). Tube B was mixed at 4°C for 2 hr after which the supernatant was transferred to tube A, this was mixed at 4°C for a further 2 hr. The supernatant from tube A was then transferred to a fresh tube (C) and stored at -80°C. IPP using JDM serum and either 150µl control [^{35}S]-methionine-labelled cell extract or the immunodepleted supernatants (C) were completed as described for IPP using [^{35}S] methionine.

IPP with mouse monoclonal anti-NXP-2 and immunodepletion experiments

Preliminary data from Oddis *et al* and Targoff *et al* have described autoantibodies to the MJ antigen, which is a 140-142 kDa protein in JDM (222, 228). The MJ target has recently been identified as nuclear matrix protein NXP-2, also termed MORC-3 (228). Immunodepletion experiments were performed to investigate the identity of the p140 target in this UK JDM cohort, which is likely to be the same as the previously identified MJ autoantigen. Therefore, immunodepletion experiments were perfomed using different reference anti-p140-JDM-positive sera and a commericial mouse monoclonal anti-NXP-2 antibody. Ten µl of anti-p140 positive sera or 50 µl of commercial mouse antibody to NXP-2 (MORC3) (Medical & Biological Laboratories, Nagoya, Japan) were mixed with 100 µl of prewashed protein G Dynabeads (Dynal, Liverpool, UK) in sodium phosphate (ph 8.1, 0.1 M) at room temperature for 30 min. The antigens were immunoprecipitated as described for IPP, using [^{35}S] methionine labelled K562 cell extract. Immunodepletion was performed to ascertain whether the

IPP pattern observed with anti-p140 positive JDM sera and the commercial antibody to NXP-2 were due to precipitation of the same antigen. Cell extracts were depleted of autoantibody targets using a reference anti-p140 positive JDM sera and normal serum (NS) as a negative control. In brief, duplicate samples each containing 10 mg protein A sepharose beads (when preparing pre-depleted p140 cell extract for IPP with commercial anti-NXP-2, 150 µl prewashed protein G Dynabeads were used) in 1 ml IPP buffer and 50 µl anti-p140 serum (or 50 µl anti-p155/140 serum) were mixed at room temperature for 30 min. The beads were washed in IPP buffer and 1 tube (A) was placed on ice whilst 120 µl [^{35}S] methionine labelled K562 cell extract and 380 µl IPP buffer was added to tube (B). Tube B was mixed at 4°C for 2 h, the supernatant was then transferred to the corresponding tube A, which was then mixed at 4°C for a further 2 h. The supernatant from the corresponding tubes (A) i.e. p140 antigen depleted cell extract were stored at -80°C. IPP with depleted cell extracts were completed using 50 µl commercial anti-NXP-2, and different anti-p140 sera (10 µl).

IPP Western blotting with mouse monoclonal anti-NXP-2

As well as immunodepletion experiments, immunoprecipitation western blotting studies using mouse monoclonal anti-NXP-2 antibodies were also performed. Forty µl of reference sera (anti-p140-JDM sera, anti-p155/140-JDM sera, anti-Mi-2-JDM sera) and 40 µl of normal sera were incubated with washed Sepharose protein A beads. Samples were centrifuged and the supernatant removed. Samples were washed twice with 1 ml triethanolamine and the supernatant removed. Samples were cross-linked and incubated with 1 ml 5mM BS^3 in triethanolamine (2.86 mg / ml – 25 mg into 8.74 ml), centrifuged and the supernatant removed. Samples were further incubated with 1 ml 50mM Tris-Cl and then washed three times with PBS and twice with IPP buffer prior to the addition of 1 ml unlabelled K562 cell extract. Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer followed by TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in 80 µl SDS sample buffer (Sigma, UK). After heating, proteins were fractionated by 10% SDS PAGE.

Nitrocellulose membrane and blotting paper was soaked in Transfer Buffer (Trizma Base, sodium acetate, EDTA, 0.1% SDS and 20% methanol). A sandwich of blotting paper, gel, nitrocellulose and blotting paper was placed in a transfer cage. Proteins were transferred to nitrocellulose membrane following electrophoresis. The nitrocellulose membrane was washed with Ponceau stain, rinsed with water and air-

dried overnight. The membrane was washed with 0.05% PBS-Tween followed by blocking solution (10% milk powder in PBS-Tween – 10 grm in 100 ml, pH 7.2). The membrane was then incubated with primary antibody (commericial mouse monoclonal anti-NXP-2 antibody – 1:200 dilution – 50 µl in 10 ml block solution) for 1 hr. The membrane was then washed in PBS-Tween, and then incubated with secondary anti-mouse IgG antibody (AbCam, UK), at 1:10,000 dilution in block solution – 50 µl in 50 ml, pH 7.2, for 1 hr. The membrane was washed again with PBS-Tween and water, and then air-dried.

6.2.3 Statistical analysis

The frequencies of clinical features were compared using the Chi-squared test with Yates' continuity correction or the Fisher's exact test for groups with small numbers. Where data was not normally distributed the Mann-Whitney-U test was used to compare continuous data. Median values (inter-quartile ranges) were expressed where appropriate and P values<0.05 were considered significant. P values (P_{corr}) were adjusted using the Bonferroni correction. SPSS for Windows (version 14) (for clinical data) was used to perform statistical analysis.

6.3 Results

6.3.1 Anti-p155/140: autoantibodies targeting a 155/140 kDa doublet protein in juvenile dermatomyositis

Serological results

Following IPP, sera from a number of JDM patients recognised two distinct proteins forming a doublet with molecular weights of 155 kDa and 140 kDa (see Figure 16). The same pattern was observed in a subset of adult DM patients (see Chapter 4, Figure 13 and this chapter, Figure 16). Non-specific weak nuclear patterns were observed on IIF between anti-p155/140 patients (data not shown).

The immunodepletion results support the co-identity of the p155/140 kDa doublet precipitated by sera from both JDM and adult DM groups (see Figure 17). When the cell extracts were pre-depleted with normal serum, no targets were removed from the extract and the 155 kDa and 140 kDa autoantigens were still precipitated by juvenile and adult sera. However, when the cell extract was pre-depleted with either juvenile or adult anti-p155/140 positive sera, the autoantigens were no longer detectable in juvenile or adult anti-p155/140 positive sera respectively. This provided

good evidence that the sera from JDM and adult DM contained the same autoantibody specificity.

Frequency and clinical features of anti-p155/140 autoantibodies

From 116 juvenile myositis sera, 27 (23%) had anti-p155/140 autoantibodies. Information on the degree of skin involvement and other selected clinical features are outlined in Table 18. There was a higher frequency of males in the anti-p155/140 positive children compared to anti-p155/140 negative children (P_{corr}=0.04). Anti-p155/140 positive JDM patients had an increased frequency of skin lesions (Gottron's papules P_{corr}=0.027, ulceration P_{corr}=0.045) with a wider distribution of cutaneous involvement, particularly over the small joints (P_{corr}<0.001) and large joints (P_{corr}=0.027). Cutaneous oedema was also more frequent in anti-p155/140-positive children (P=0.013) but this was not significant following multiple corrections. Overall, there was no significant difference in those with elevated muscle enzymes at diagnosis or during disease course or those with an abnormal MRI or muscle biopsy between anti-p155/140 positive and negative groups (data not shown). However not all children had data on this, in particular some did not have a MRI or biopsy performed. There was a trend towards lower CMAS (lower values indicate more severe weakness) and higher PGA in anti-p155/140 positive children at baseline and during follow up, although this did not reach statistical significance. The frequency of other clinical signs including lipoatrophy, arthritis, Raynaud's, sclerodermatous skin changes, dysphagia, mouth ulcers or alopecia was not significantly different between children with or without anti-p155/140. There was no history of malignancy in the entire JDM cohort during the follow-up period.

Figure 16: Immunoprecipitation of p155/140 kDa autoantigens. 10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract. Sera used for immunoprecipitation include Lanes 1-3; adult anti-p155/140 positive serum, Lanes 4-6; juvenile anti-p155/140 positive serum, Lane 9; juvenile anti-Jo-1 (histidyl tRNA synthetase) positive serum, Lane 10; juvenile anti-Mi-2 positive serum. Positions of the p155 and p140 antigens are indicated on the left.

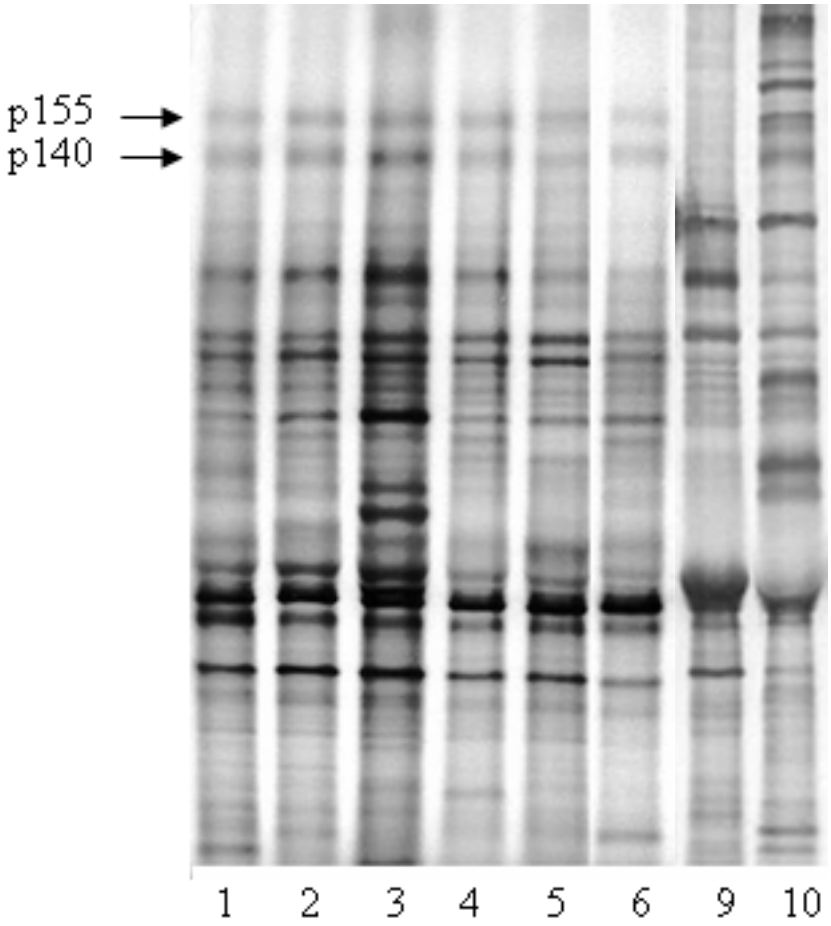


Figure 17: Immunodepletion experiments - Autoradiogram of 10% SDS-PAGE of immunoprecipitates using either anti-p155/140-adult DM sera or anti-p155/140-JDM positive sera. Immunoprecipitation was performed with control [^{35}S]-methionine-labelled cell extract or [^{35}S]-methionine-labelled cell extract depleted with either normal serum (NS), adult positive anti-p155/140 serum or JDM positive anti-p155/140 serum. The bands corresponding to the p155 and p140 autoantigens are indicated.

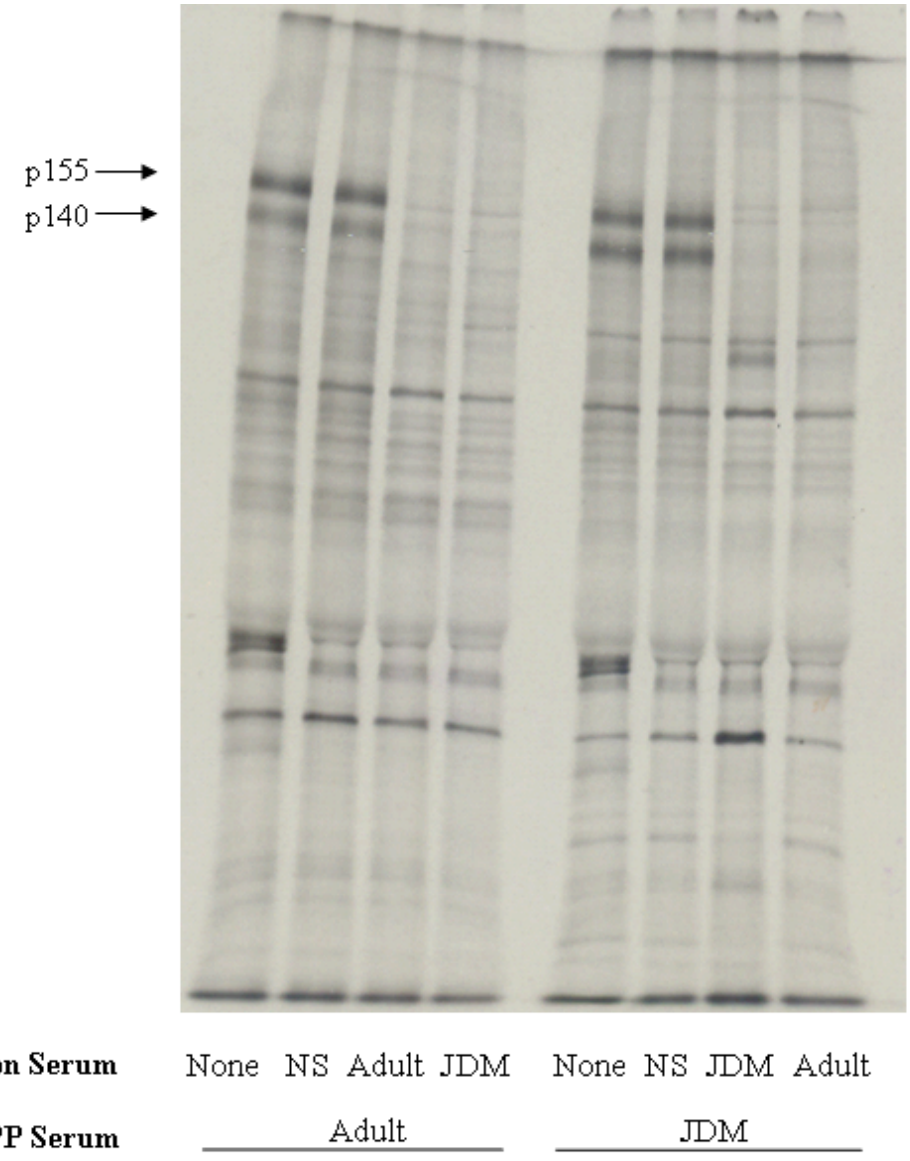


Table 18: Selected clinical associations of anti-p155/140 autoantibodies in JDM patients

	Anti-p155/140		
	Positive (n=27)	**Negative (n=89)**	P_{corr}
Age at diagnosis, median (IQR)	6 years (4, 10)	7 years (5, 10)	
Male:Female	44.4	20.2	0.04
Type of skin lesion			
Gottron's papules	100	76.1[#]	0.027
Ulceration	51.9	21.3	0.045
Oedema	63	33.7	*NS**
Calcinosis	11.1	23.6	*NS*
Lipoatrophy	17.9	14.3	*NS*
Distribution of skin lesion			
Periorbital	92.6	65.9[#]	*NS*
Periungal	81.5	59.1[#]	*NS*
Trunk	6 (22.2)	10[#] (11.4)	*NS*
Small joints	100	62.5[#]	<0.001
Large joints	77.8	43.2[#]	0.027
Muscle disease[∂]			
Baseline CMAS, median (IQR)	36 (13.8, 48)	44 (35, 50.5)	*NS*
Lowest CMAS, median (IQR)	36 (13.8, 46.8)	43 (29, 49.0)	*NS*
Baseline PGA, median (IQR)	4.7 (2.0, 7.1)	2.8 (1.1, 5.0)	*NS*
Highest PGA, median (IQR)	5.5 (2.5, 7.1)	3 (1.2, 5.1)	*NS*

Values in percentages unless otherwise indicated. [#]Data on 88 children. [∂]Not all patients had data available for each clinical feature. CMAS (childhood myositis assessment scale: 0-53) at baseline and lowest (worst) score during follow-up. PGA (physician's global assessment: 0-10) at baseline and highest (worst) score during follow-up.

*$P_{(uncorr)}$=0.013

6.3.2 Anti-p140: autoantibodies targeting a nuclear matrix protein NXP-2 in juvenile dermatomyositis

Serological results

Identification of anti-p140 autoantibodies in JDM sera

Following IPP, sera from a number of JDM patients recognized a distinct protein band with a molecular weight of ~140 kDa (see Figure 18). No anti-p140 positive sera were found to immunoprecipitate any other known MSAs or MAAs. A weak non-specific nuclear pattern or in some cases a negative ANA was observed on IIF in all anti-p140 sera. Anti-p140 was not detected in any of the juvenile disease control sera or healthy adult control sera.

Immunodepletion experiments using anti-140 and anti-p155/140 JDM sera

Immunodepletion confirmed anti-p140 sera target a different protein to JDM sera that recognises the p155/140 doublet protein (see Figure 19, lanes 1-4) showing respective bands are still present following IPP).

The IPP Western blotting with mouse monoclonal anti-NXP-2 studies were negative (did not yield any results). No reference p140 bands were recognised following incubation with primary mouse monoclonal antibody anti-NXP-2 and secondary anti-mouse IgG antibody. This may be because of loss of immunoreactivity of the p140 autoantigen following transfer from SDS-PAGE to nitrocellulose membrane.

Confirmation of the p140 autoantigen

The results suggest that the p140 protein targeted by JDM sera in this study has the same identity as the MJ antigen, NXP-2 (228). Using a commercial antibody raised against NXP-2, IPP resulted in the precipitation of a band with the same molecular weight and IPP pattern as that observed in anti-p140 positive JDM sera (see Figure 20, lane 3). When cell extract was pre-depleted with JDM anti-p140 positive sera, the IPP band present with commercial anti-NXP-2 was no longer detectable (Figure 20, lane 4). The immunodepletion results supported the co-identity of the p140 protein precipitated by different anti-p140 positive juvenile sera. Figure 19 (lanes 5-6) shows an example where the 140 kDa band is no longer detectable following IPP with anti-p140 sera (1) and (2) using pre-depleted reference p140 cell extract.

Clinical associations of anti-p140 autoantibodies

Of 162 juvenile myositis sera serotyped by IPP, 37 (23%) had anti-p140 autoantibodies. This autoantibody specificity was found exclusively in JDM patients,

with a frequency of 27%. Anti-p140 was not detected in any overlap JDM-SSc sera. Selected clinical features of anti-p140 positive patients compared to the overall anti-p140 negative JDM cohort are shown in Table 19. Table 20 shows a comparison between anti-p140, anti-p155/140 and MAA positive cases. There was no significant difference in female:male ratio, age at onset or diagnosis and disease duration (from disease onset to time of this study) between anti-p140 positive and negative cases. Overall there was no significant difference in the presence of Gottron's lesions, skin ulceration and oedema or the distribution of rash except anti-p140 positive children had no rashes on the trunk compared to anti-p140 negatives (P_{corr}=0.02). Anti-p140 positive cases had a significant association with the presence of subcutaneous calcinosis compared to anti-p140 negative patients (P_{corr}<0.005, odds ratio 7.0, 95% CI 3.0-16.1). There was no significant difference between other clinical features when comparing anti-p140 positives *versus* negatives including baseline CK, CMAS, childhood health assessment questionnaire (CHAQ), physician's global assessment scale (PGA), and the presence of arthritis, Raynaud's, dysphagia, mouth ulcers and alopecia (data not shown).

Possible clinical differences were noted when anti-p140 positive patients were compared to anti-p155/140 positive patients (Table 18). Overall, age of onset or diagnosis and disease duration (anti-p140 positives, median 48 months IQR 34, 72 *versus* anti-p155/140 positives, median 52 months, IQR 36, 84) was similar between the autoantibody groups. Compared with anti-p155/140 positive children, anti-p140 positives again had an association with calcinosis, 54% versus 14%, P_{corr}=0.015 OR 7.1 (2-25). In contrast, anti-p155/140 positives compared with the anti-p140 positive group had a higher frequency of ulceration and cutaneous oedema however this result was not significant after correcting for multiple comparisons. The distribution of rash was wider on the trunk P_{corr}<0.005 and over the small joints (P_{corr}=0.05 OR 13.5 95% CI 2-113) in anti-p155/140 compared with anti-p140 positive cases. At the time of diagnosis, anti-p140 compared to anti-p155/140 positive children had a non-significantly lower CK (median 202, IQR 76, 2142 versus median 571 IQR 234, 2495) and LDH (median 845 IQR 710, 1620 versus median 1171 IQR 736, 1647). In addition, anti-p140 positives had a higher CMAS at diagnosis compared to anti-p155/140 positives (median 42, IQR 23, 49 versus median 16, IQR 7, 38) (not significant after adjustment for multiple comparisons). There was a non-significant trend towards a lower baseline CHAQ and PGA in the anti-p140 group compared to the anti-p155/140 group; median CHAQ 1.31 (0.75, 1.63) versus 1.63 (0.78, 2.34), median PGA 5 (3, 7.4) versus 7.3 (5, 7.8) respectively. However, this data needs to

be interpreted in the context that a small number of patients in either group had this data recorded at diagnosis.

Figure 18: Immunoprecipitation of p140 autoantigens

Autoradiogram of 10% SDS PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract. Sera used for immunoprecipitation include Lane 1; normal serum, Lane 2; anti-p155/140 positive JDM serum, Lanes 3-14; different anti-p140 positive JDM sera.

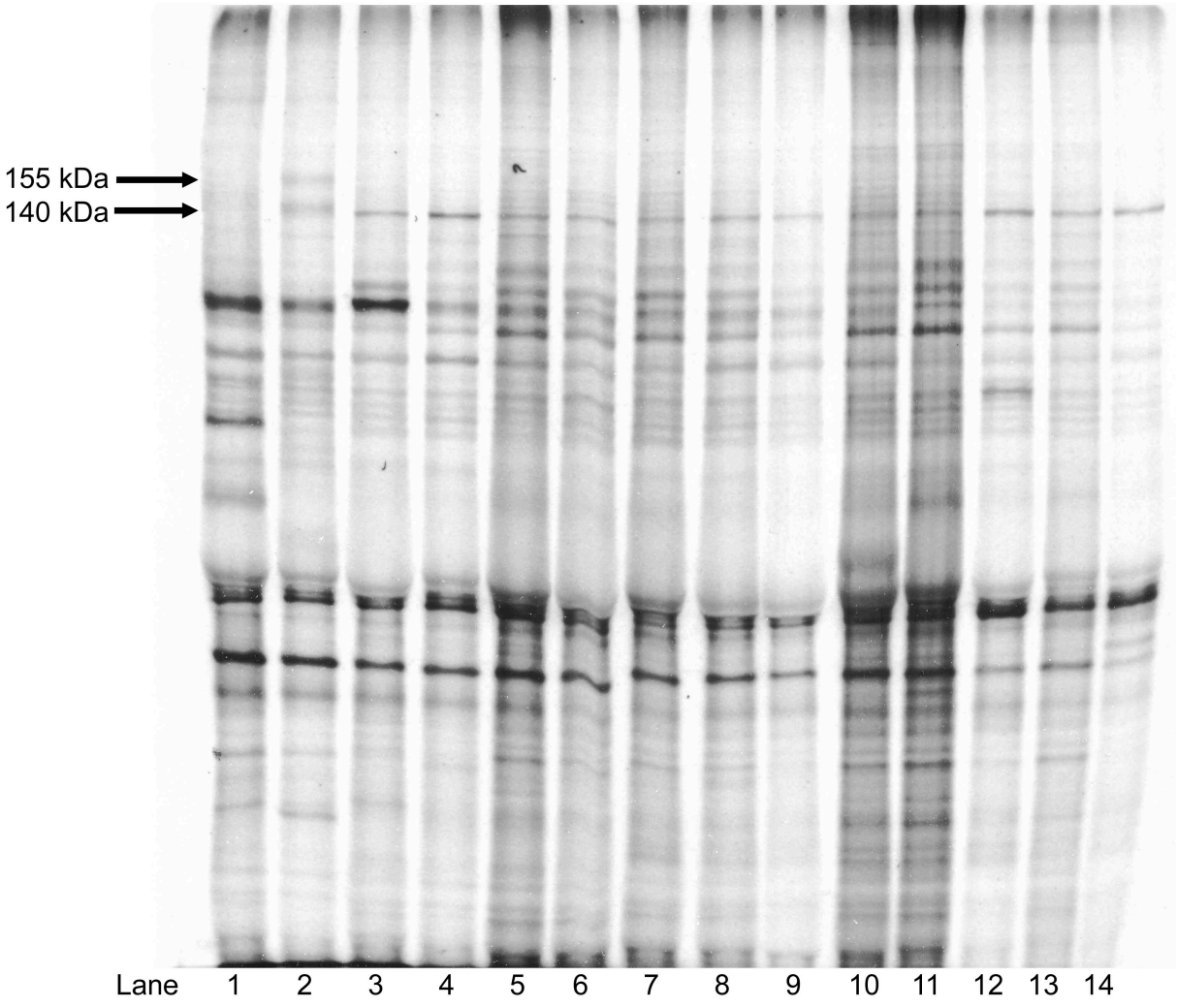


Figure 19: Immunodepletion experiments with anti-p140 and anti-p155/140

Autoradiogram of 10% SDS-PAGE of immunoprecipitates using different anti-p140 positive JDM sera (lanes 1-2 and 5-6) and anti-p155/140 positive JDM sera (lanes 3-4). Immunoprecipitation was performed with [^{35}S] labelled K562 cell extract pre-depleted with either reference anti-p140 positive JDM serum or reference anti-p155/140 positive JDM serum.

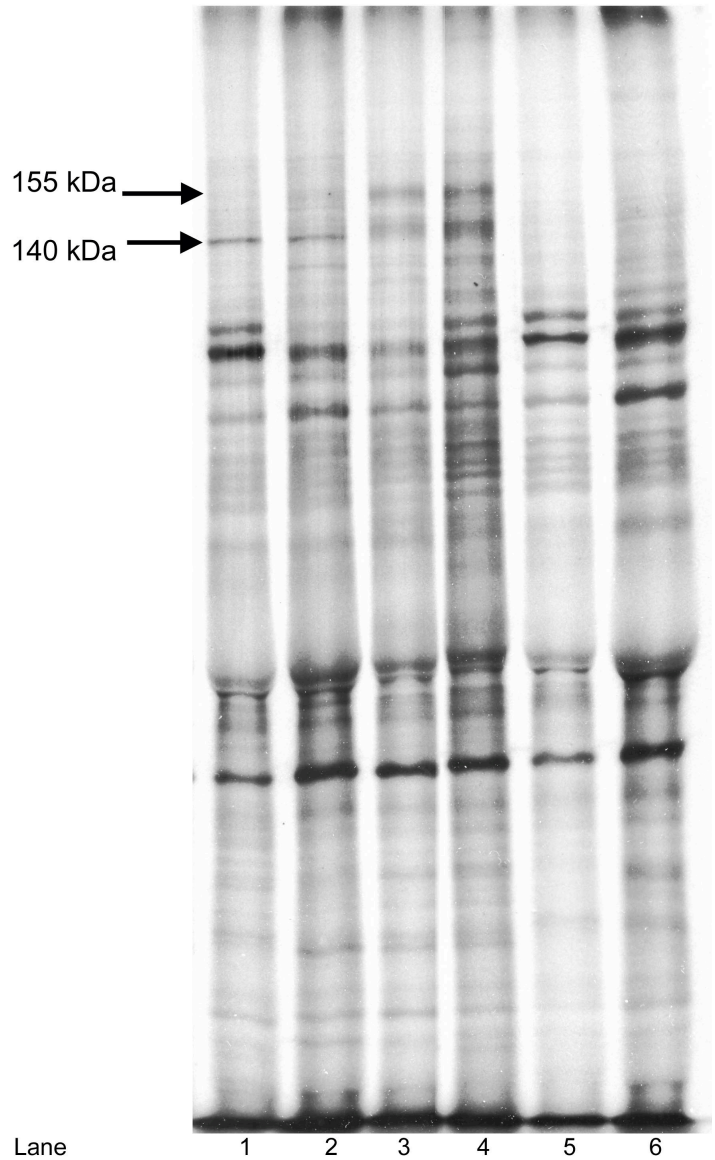


Figure 20: Immunoprecipitation and immunodepletion of mouse monoclonal nuclear matrix protein NXP-2

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract using normal sera (NS) (lane 1), reference anti-p140 positive JDM sera (lane 2) and commercial anti-NXP-2 (lane 3). Lane 4, immunoprecipitation using commercial anti-NXP-2 with [^{35}S] labelled K562 cell extract pre-depleted with reference anti-p140 positive JDM serum.

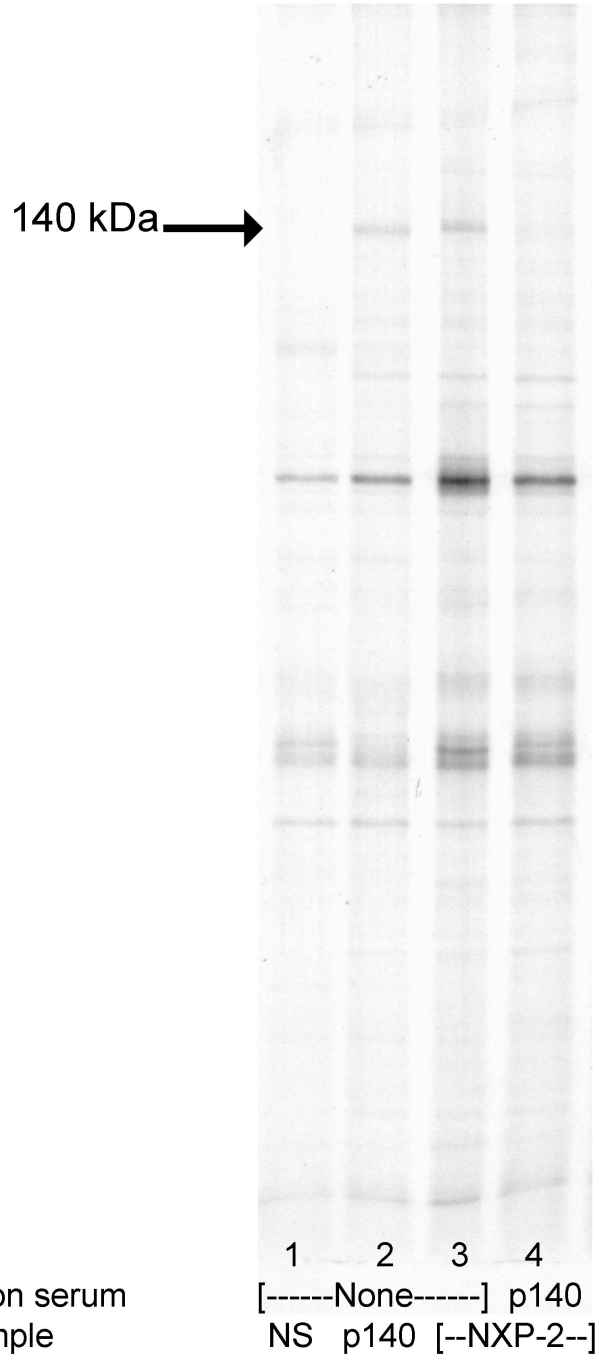


Table 19: Selected clinical associations of anti-p140 positive JDM patients compared with JDM patients overall (anti-p140 negative) and with anti-p155/140 positive JDM patients *

	Anti-p140 positive (n=37) **	All JDM anti-p140 negative (n=125)**	Anti-p155/140 positive (n=28)
Age at onset (yrs)	6	6	6
Median (IQR)	(2, 10)	(4, 9)	(4, 10)
Age at diagnosis (yrs)	7	7	7
Median (IQR)	(4.5, 10.3)	(4, 10)	(4, 10)
Female	72	74	57
Type of skin lesion			
Gottron's papules	85	82	100
Ulceration	34	23	57
Oedema	35	35	64
Calcinosis	54 ¶	15	14
Lipoatrophy	18	13	18
Distribution of skin rash			
Periorbital	79	69	96
Periungal	64	67	86
Small joints	67 #	72	96
Large joints	52	51	75
Trunk	0 ##	18	32

* Values are the percentage of patients, unless otherwise stated.

** Not all patients had the clinical data available for each feature.

P_{corr}: corrected P values, OR: odds ratio (with 95% confidence intervals).

¶ P_{corr}<0.005, OR 7.0 (3.0 - 16.1) versus all JDM anti-p140 negative patients and P_{corr}=0.015, OR 7.1 (2-25) versus anti-p155/140 positive patients.

P_{corr}=0.05 OR 13.5 (2-113) anti-p155/140 positive patients versus anti-p140 positive patients.

P_{corr}=0.02 versus all JDM anti-p140 negative patients and P_{corr}<0.005 versus anti-p155/140 positive patients.

Table 20: Clinical associations of anti-p140-JDM patients compared with anti-p155/140 positive-JDM, and MAA-positive- JDM patients *

	Anti-p140 positive (n=37) **	Anti-p155/140 positive (n=28) **	MAA-positive (n=23) **
Age at onset (yrs)	6	6	6
Median (IQR)	(2, 10)	(4, 10)	(4, 10)
Age at diagnosis (yrs)	7	7	9
Median (IQR)	(4.5, 10.3)	(4, 10)	(5, 11)
Female	72	57	78
Type of skin lesion			
Gottron's papules	85	100 ¶	65
Ulceration	34	57 ¶¶	13
Oedema	35	64 α	17
Calcinosis	54 αα	14	22
Lipoatrophy	18	18	35
Distribution of skin rash			
Periorbital	79	96	35
Periungal	64	86 λ λ	57
Small joints	67	96 #	65
Large joints	52	75 ##	30
Trunk	0	32 ###	17
Other features			
Arthritis	49	39	65
Sclerodermatous features	3	4	61 ə
Raynaud's phenomenon	11	11	48 ə ə
Dysphagia	31	43	35
Mouth ulcers	29	32	4 γ
Alopecia	37	39	26

Key for Table 20

* Values are the percentage of patients, unless otherwise stated.

** Not all patients had the clinical data available for each feature.

P_{corr}: corrected *P* values

¶ P_{corr}=0.01, anti-p155/140-positives versus MAA-positives

¶¶ P_{corr}=0.03, anti-p155/140-positives versus MAA-positives

α P_{corr}=0.02, anti-p155/140-positives versus MAA-positives

αα P_{corr}=0.01, anti-p140-positives versus anti-p155/140-positives / MAA-positives

λ P_{corr} <0.001, anti-p155/140-positives versus MAA-positives

P_{corr}=0.045, anti-p155/140-positives versus MAA-positives / anti-p140-positives

P_{corr}=0.03, anti-p155/140-positives versus MAA-positives

P_{corr}=0.01, anti-p155/140-positives versus MAA-positives and anti-p140-positives

ə P_{corr}<0.001, MAA-positives versus anti-p140 / anti-p155/140-positives

əə P_{corr}<0.001, MAA-positives versus anti-p140 / anti-p155/140-positives

λ λ P_{corr}=NS (*P*=0.045, anti-p155/140-positives versus MAA-positives / anti-p140-positives

γ P_{corr}=NS (*P*=0.04, anti-p155/140 and anti-p140-positives versus MAA-positives

6.4 Discussion

As described in this thesis and in previous studies there is now increasing evidence that MSAs are associated with clinical subsets in the adult IIM spectrum. The work described in Chapter 5 and 6 suggests serological profiles may also be associated with clinical phenotypes in JDM. Previously, myositis autoantibodies were detected infrequently in JDM with anti-Mi-2 having the strongest association (see Chapter 5) (125, 127). Therefore, detection and characterisation of novel MSAs in JDM may identify distinct clinical subsets within this disease group. Identification of new autoimmune markers may help clinicians predict clinical outcomes and lead to further insights in disease pathogenesis.

There have been reports of novel protein targets including p140 kDa and p155 kDa polypeptides in both adult and juvenile DM. Two reports have described autoantibodies targeting a 140 kDa protein (anti-MJ) and a 155/(140) kDa protein in US JDM patients (143, 222). In adult DM, anti-p155/140 autoantibodies are associated with a history of malignancy (see Chapter 4) (143, 157, 158). In the study described in Chapter 4 (see Section 4.3.4, Table 12), anti-p155/140 autoantibodies were also detected in adults with CADM with no history of cancer and although the numbers are small, this group of adults tended to be younger. In this cohort of children with JDM, no malignancy has been reported to date in those with anti-p155/140 autoantibodies; long term follow up studies will be required to ascertain whether there is any association with malignancy later in life in this group. In addition, validation of anti-p155/140 in larger groups of both adult and juvenile DM is required. An important finding of this study is the demonstration by immunodepletion that anti-p155/140 autoantibodies appear to target the same autoantigen in both adult and juvenile DM. Targoff *et al* described anti-p155 (in most cases with a second weaker 140 kDa band) in approximately 30% of their JDM population (143). The clinical specificity of this cohort was not described. This study describes detailed clinical features of JDM patients with autoantibodies targeting p155/140 and confirms they have similar cutaneous clinical associations to adults with the same autoantibody specificity (as described in Chapter 4, Section 4.3.4, Table 11). Like adult DM cases with anti-p155/140 these children have more extensive skin involvement including Gottron's papules over a wider distribution. Anti-p155/140 autoantibodies also appear to define a subset of JDM with more peripheral oedema and skin ulceration. There was a trend towards lower baseline and worst ever CMAS, and higher PGA in anti-p155/140 positive JDM compared to negative patients, although this was not statistically significant. In summary, anti-p155/140

autoantibodies occur frequently in JDM and the preliminary findings suggest that they may identify patients with more severe skin and possibly muscle disease. Identification of this autoantibody at diagnosis may help predict the clinical course.

In this chapter, a further novel autoantibody specificity in the JDM UK cohort is described. Anti-p140 autoantibodies form a major serological subset in juvenile myositis found exclusively in JDM and not detected in any JDM-SSc overlap cases. Anti-p140 positive sera recognised the same polypeptide and did not immunoprecipitate any other known myositis autoantigens, including the anti-p155/140 autoantibody. Combining the data from Study 1 and 2 in this chapter, anti-p140 and anti-p155/140 autoantibodies are detected in approximately 40% of JDM cases, in contrast to a much lower frequency of anti-Mi-2 and other myositis-specific or associated autoantibodies as published previously (10, 125, 127, 219).

The confirmation of a further serological subset in JDM appears to have important clinical implications. Anti-p140 has a significant association with the presence of calcinosis when compared to the overall juvenile myositis cohort. In addition, anti-p140 and anti-p155/140 appear to define JDM into two serological subsets with more homogeneous clinical features. Combined with the data from Study 1 in this chapter, anti-p155/140 positive children appear to have a wider distribution of skin disease, more cutaneous complications including oedema or ulceration and possibly overall higher disease activity but a significantly lower frequency of calcinosis compared to anti-p140 positive children. The clinical differences observed between anti-p140, anti-p155/140 positives and patients without either of these specificities are not explained by differences in time between disease onset or disease duration. This is an interesting observation because factors suggested to influence the development of calcinosis are persistent active disease including chronic cutaneous inflammation (185, 201, 229). The association between serotype and clinical phenotype suggests that the targeted autoantigens p140 and p155/140 may play role in the pathogenesis of skin and soft tissue complications in JDM. Based on previous work that showed an association with the TNFα-308A allele, an increased production of tumour necrosis factor-α and calcinosis (201); future studies to investigate for other potential susceptibility genes including TNF polymorphisms in JDM patients with anti-p140 or anti p155/140 autoantibodies would be of major interest.

The p140 protein targeted by an autoimmune response in our JDM cohort study is consistent with nuclear matrix protein NXP-2, the MJ autoantigen described in preliminary reports in a US JDM cohort, and most recently in an Argentine JDM patient study by Espada *et al* (222, 225, 228). In the latter study, RNA and protein immunoprecipitation and immunoblotting tested 64 patients with juvenile IIM for autoantibodies. The anti-MJ autoantibody was detected exclusively in 25% of cases, a similar frequency to the anti-p140 autoantibody found in the UK JDM cohort study described here. Clinical features of anti-MJ autoantibodies suggest this specificity is a marker of disease severity, although the numbers were small and no direct comparisons with other serological groups was made. Muscle contractures (44%) and lipoatrophy (44%) were more frequent in anti-MJ-positive cases. This specificity was detected predominantly in pure JDM cases with proximal weakness and classic skin changes. The frequency of calcinosis was 31% and cutaneous vasculitis (with skin ulceration) 38% respectively. Clinical similarities with the anti-MJ and anti-p140 autoantibody are noted between this Argentinean cohort and the UK JDM cohort. The main differences, in particular the prevalence of lipoatrophy, may represent variations in clinical evaluation and length of follow-up for each study (225).

In the study by Espada *et al* 22% of juvenile patients (all JDM) were positive for the anti-p155/140 autoantibody, again a similar number to that detected in the work in this chapter. All children had proximal muscle weakness with a high prevalence of muscle contracture, and the majority has classic DM skin disease. There was also a relatively high frequency of cutaneous vasculitis with persistent ulceration (57%) (225), the same frequency noted in anti-p155/140-positive children from the UK cohort study (see Table 18 and 19).

A further clinically important complication of JDM is lipodystrophy or lipoatrophy (a loss of subcutaneous fat, which is either localised or generalised affecting the face, trunk and limbs). Previous studies have reported prevalence rates between 12-40% (24, 230, 231). In a recent study, Bingham *et al* reported a lower frequency of lipodystrophy of 8% in a tertiary referral centre juvenile IIM registry (232). In the Bingham *et al* study, generalised lipodystrophy was shown to be associated with the anti-p155 (anti-p155/140) autoantibody (143), as well a chronic disease course, muscle atrophy, joint contractures, and interestingly calcinosis (232). In this UK cohort study, the overall frequency of lipodystrophy / atrophy was 14% (see Chapter 5, Section 5.3.2, Table 16), although the prevalence of this complication in anti-p155/140 and anti-p140 positive children was not significantly different compared to

the rest of the cohort. Interestingly, lipoatrophy was seen in a relatively higher frequency of children who were positive for MAAs, however this did not reach statistical significance. A potential reason for the difference in lipoatrophy associations between this study and the work by Bingham *et al* (232) is the way respective clinicians define lipodystrophy or lipoatrophy. Furthermore, the relatively higher frequency of lipoatrophy in MAA-positive children may be because this overall autoantibody group was associated with JDM-scleroderma overlap.

Larger collaborative studies are required to evaluate further the clinical specificity of both anti-p155/140 and anti-p140 autoantibodies in juvenile myositis populations. Work to date and the work presented in this chapter suggest that these two specificities are very important autoantibody systems with pathogenic, clinical and prognostic implications. When grouped together these two serological subsets are positive in between 40-50% of JDM cases, and appear to define those children with a more severe disease course including an association with widespread skin lesions, skin ulceration, oedema, lipodystrophy, calcinosis and worse muscle disease with muscle contracture (222, 225, 232, 233) (and Chapter 6).

The identity of the p155 kDa and p140 kDa doublet protein requires investigation. It is likely that the p155/140 autoantigen identified in this work is the same as the p155/140 doublet proteins identified in recent studies (143, 157, 158), although immunodepletion or reverse IPP blotting studies with prototype sera are required for further confirmation. A preliminary report has identified the target of the anti-p155 autoantibodies as transcriptional intermediary factor 1-gamma (TIF1-γ) (159). This nuclear protein is a member of a novel family of transcriptional coregulator-encoding genes, which function in cell differentiation and development (181). As yet, it is unclear what relationship the p140 protein (as part of the p155/140 doublet) has to the identified p155 TIF1-γ autoantigen i.e. is this a separate protein, which forms part of a multi-protein complex, or is it simply a cleaved or modified fragment of the same protein.

Augmented expression myositis-specific autoantigens in diseased muscle and cancer tissue suggests a new paradigm for pathogenesis of IIM (107, 137). It is not clear why a tumour-associated autoantigen in adult DM should be recognised in JDM, particularly because there appears to be no association malignancy. However, other clinical features including the degree of skin disease in anti-p155/140 positive children and adults are very similar. Longitudinal prospective studies are required to

investigate whether this autoantibody in juvenile disease persists into adulthood and if so are they clinically significant. Perhaps the observation that the same autoantigen system is expressed in younger DM adults without cancer as well JDM suggests that some perturbation of p155/140 expression in proliferating cells combined with a more efficient anti-cancer response by a younger immune system may be a unifying mechanism to explain why it is targeted by an autoimmune response in children and adults with DM.

The p140 autoantigen NXP-2 has nuclear matrix binding, RNA-binding, and coiled-coil domains that are structurally separated, which implicates a role in diverse nuclear functions including regulation of transcription and RNA metabolism (228, 234). The other dermatomyositis-specific autoantigen targets in children and adults; Mi-2 and p155/140 (p155 – transcription intermediary factor 1-gamma) (159, 181) are nuclear proteins that also mediate gene transcription. It is of further interest to note that autoantibodies to small ubiquitin-like modifier enzyme (SAE), which is involved in post-transcriptional modification termed sumoylation, have been described in adult dermatomyositis (see Chapter 4, Section 4.3.2) (160). It is interesting that anti-SAE autoantibodies were not detected in any sera from the UK JDM cohort (Chapters 5 and 6), particularly because NXP-2 has been shown to be a sumoylation target involved in transcriptional repression (235). However, this observation may suggest shared pathogenic mechanisms in both juvenile and adult dermatomyositis.

In conclusion, anti-p155/140 and anti-p140 are clinically important serological markers found in a high frequency of JDM patients that are associated several disease complications, which confers significant morbidity. In the future, routine testing of these novel autoantibodies at disease onset could have prognostic value and identify those children at risk of more severe disease, which may influence management. Furthermore, increasing our understanding of autoimmune targets and their relationship to clinical phenotype in juvenile myositis may provide further insight into pathogenic pathways, which in turn will stimulate new therapeutic approaches.

6.5 Appendix

I. Acknowledgements

I would like to thank Mrs J Dunphy and Mrs P Owen for their assistance and guidance in performing ANA IIF on the juvenile myositis samples. Thank you to Dr Zoe Betteridge for her guidance with my immunoprecipitation, immunodepletion and western blotting studies. I would also like to thank Prof Lucy Wedderburn, Scientific Director for the Juvenile Dermatomyositis UK Cohort Study for her collaboration, guidance and support.

On behalf of the Juvenile Dermatomyositis Research Group I would like to thank all the patients and their families who contributed to the Juvenile Dermatomyositis Cohort Study. I thank all local research coordinators and principal investigators who have made this research would not have been possible. The members who contributed were: Mr Ian Roberts, The Royal Liverpool Children's Hospital, Alder Hey, Liverpool, and Booth Hall Children's Hospital, Manchester; Dr Eileen Baildam, Booth Hall Children's Hospital, Manchester and now Alder Hey, Liverpool,; Dr Phil Riley, Booth Hall Children's Hospital, Manchester; Mrs Janis Scott and Dr Clive Ryder, Birmingham Children's Hospital, Birmingham; Mrs Gillian Jackson and Dr Sue Wyatt, Leeds General Infirmary, Leeds; Ms Elizabeth Camp and Dr Janet Gardner-Medwin, The Royal Hospital for Sick Children, Yorkhill, Glasgow; Mrs Alison Swift, Dr Helen Foster and Dr Mark Friswell, The Royal Victoria Infirmary, Newcastle; Mrs Elizabeth Hutchinson and Dr Helen Venning, Queens Medical Centre, Nottingham and Dr Clarissa Pilkington, Dr N Hasson and Ms Sue Maillard, Great Ormond Street Hospital, London. I thank H Varsani for technical assistance, in particular preparation of the serum samples, which were sent to the RNHRD for this study. The Arthritis Research Campaign awarded me the Barbara Ansell fellowship in Paediatric Rheumatology (grant 18136) to perform this work. I am grateful to the Arthritis Research Campaign who funded the autoantibody testing for this study as part of my fellowship.

CHAPTER SEVEN
DISCUSSION

Introduction

This chapter will outline two preliminary studies conducted alongside the main body of this thesis. The chapter will close with a general discussion of the work conducted as part of this thesis, and it's relevance to the current understanding of the relationship between pathogenesis, serological subsets and clinical phenotypes in adult and juvenile IIM. To conclude, hypothetical models of autoimmune dysregulation in distinct microenvironments and how this may lead to disease initiation and propagation in IIM, will be explored. Potential future work focusing on antigen expression at a cellular and tissue level, and the development of solid phase assays for commercial testing of novel myositis-specific autoantibodies (MSAs), will be discussed.

7.1 Other preliminary studies

7.1.1 Autoantibodies in idiopathic interstitial pneumonia

Introduction

As described in previous chapters, interstitial pneumonia is a frequent and significant manifestation of adult IIM, particularly the anti-synthetase syndrome (ASS) subset. Moreover, interstitial lung disease may be the presenting or predominant manifestation of IIM, especially in ASS "sine myositis" and clinically-amyopathic DM (29, 31, 40-42).

NSIP has been reported to be the most frequent subtype on both HRCT and lung biopsy in IIM (38). AIP, OP and UIP have also been reported and identified as patterns responsible for acute or rapidly progressive interstitial pneumonia with AIP and UIP associated with the worst prognosis (28-31, 33, 39). It is well recognised that interstitial lung involvement may be the first or predominant organ manifestation preceding muscle or skin disease, particularly in the anti-synthetase syndrome (see Section 1.5.1) and in patients with clinically-amyopathic DM (CADM) (see Section 1.5.3). Similarly, it is now recognised that interstitial pneumonia can be the major complication of systemic sclerosis sine scleroderma, particularly in association with the specific scleroderma-associated autoantibody anti-Th/To (227, 236-238).

Diffuse parenchymal lung disease is labelled idiopathic interstitial pneumonia when there is no evidence of a causative agent or coexisting connective tissue disease. The standard autoimmune profile is usually negative, even in patients who later

manifest a connective tissue disease (CTD). Therefore some patients with interstitial pneumonia may have underlying CTD characterised by the presence of specific autoantibodies not detected on routine serological testing, particularly ANA ELISA (most commonly used in routine clinical laboratories). In this pilot study, a small cohort of patients previously diagnosed as having idiopathic interstitial pneumonia have been screened for CTD-associated autoantibodies using ANA IIF and protein immunoprecipitation (IPP).

Patients and Methods

Twenty patients with a previous clinico-radiological diagnosis of idiopathic interstitial pneumonia were studied (patients from the Royal United Hospital Interstitial Lung disease clinic were invited to participate and not pre-selected). On further detailed clinical review by a Rheumatologist (HG), some patients had clinical features suggestive of an underlying CTD including a history of Raynaud's phenomenon (see Table 22).

All patients had high-resolution computerised tomography (HRCT) scans, which were re-reviewed (see Section 3.2.2). The subtype of interstitial pneumonia was categorised based on the American Thoracic Society / European Respiratory Society classification (32).

Patients' sera were analysed using ANA indirect immunofluorescence (IIF) and further characterised using protein immunoprecipitation of ^{35}S-labelled K562 cells combined with SDS-PAGE to identify specific autoantigen targets, as described previously (see Chapter 2, Section 2.3.1 and 2.3.2).

Results

For detailed results see Table 21 and see Figure 21. Six patients had a positive ANA on IIF (>1/40 titre), all with a homogeneous pattern. Of those, two patients' sera were negative on IPP, three cases had strong unidentified bands, and one patient was anti-topoisomerase positive on IPP (characteristic 100 kDa band, and confirmed by ENA ELISA). Despite two patients testing negative on ANA IIF, both had unidentified bands on IPP.

Five patients' sera with negative anti-nuclear staining but a strong cytoplasmic speckle tested positive for recognised anti-aminoacyl tRNA synthetase (ARS)

autoantibodies (one anti-Jo-1, one anti-PL-7, two anti-PL-12, and one patient with possible anti-Ha autoantibodies). See Table 22.

Discussion

In this preliminary study, 30% of patients with a previous diagnosis of idiopathic interstitial pneumonia actually tested positive for CTD-associated autoantibodies. One case tested positive for anti-topoisomerase autoantibodies, with NSIP lung disease, and clinical features of Raynaud's phenomenon. Therefore, the patient may be re-diagnosed as having systemic sclerosis sine scleroderma (major SSc organ involvement, in this case fibrotic lung disease, a positive SSc-autoantibody, without characteristic skin changes of scleroderma) (239). In two recent studies by Fischer *et al*, a subset of patients with idiopathic interstitial pneumonia had nucleolar staining patterns of ANA IIF, the majority of which were positive for anti-Th/To autoantibodies (236, 237). Anti-Th/To is associated with less sclerodermatous features and a higher risk of pulmonary hypertension and lung fibrosis (227, 239), and this autoantibody specificity should be considered in patients with these clinical manifestations.

Of those 30% with CTD-autoantibodies, the other cases were positive anti-ARS autoantibodies, in particular non-Jo-1-anti-ARS. All non-Jo-1-anti-ARS has a clinico-radiological pattern consistent with UIP, except the anti-PL-7 case, which had NSIP. As described in Chapter 3 (see Section 3.3.3), lung fibrosis is often the predominant clinical manifestation in non-Jo-1-ARS-postive patients. Furthermore, radiological patterns demonstrate more architectural distortion with pronounced traction bronchiectasis and honeycombing, and histologically fibroblastic foci. This subtype of interstitial pneumonia is synonymous with idiopathic pulmonary fibrosis (formerly termed cryptogenic fibrosing alveolitis). Therefore, it is possible a proportion of patients with idiopathic pulmonary fibrosis actually have non-Jo-1-ARS, especially anti-PL-12 autoantibodies (42, 150). Anti-PL-7 autoantibodies have also been shown to be significantly associated with interstitial pneumonia and much milder or subclinical muscle disease (145).

In summary, patients with a presumed idiopathic interstitial pneumonia may have subtle features of underlying CTD, and a negative routine autoimmune panel should not dissuade clinicians to consider more novel serological markers of autoimmunity in some. Moreover, even in patients with UIP lung disease, a negative ANA but a strong cytoplasmic pattern on IIF should prompt more detailed investigation. A

proportion of these patients may be a "*formes fruste*" of an underlying CTD, in particular anti-synthetase syndrome where lung disease is the only or predominant manifestation. Historically, the general consensus has been patients with idiopathic lung fibrosis have a limited response to immunomodulatory therapy, whereas patients with CTD-associated lung disease do appear to respond or stabilise with corticosteroid and immunosuppressive therapies. Thus, early identification of this group of patients will influence treatment strategies and long-term prognosis.

Part of this preliminary study has been published in abstract form:

Gunawardena H, Betteridge Z, Owen P, McHugh NJ, Foley NM. Novel markers of connective tissue disease in patients with idiopathic interstitial pneumonia. European Respiratory Journal 2008;32:320s.

Table 21: Serological results in idiopathic interstitial pneumonia patients

Patient	Age	IIP subset	ANA on IIF	IPP
R23840	61	NSIP	1/160 homogeneous	Negative
R23839	73	NSIP	Negative	Non-specific bands,
R23838	74	NSIP	Negative with strong cytoplasmic speckle	Anti-Jo-1
R23837	75	UIP	1/40 homogeneous	Negative
R23843	66	Indeterminate	Negative	Negative
R23842	46	NSIP	Negative	Negative
R24103	61	UIP	Negative	Unknown 112 kDa band
R24101	58	NSIP	Negative but strong cytoplasmic speckle	Anti-PL-7
R24102	53	RBILD	1/40 homogeneous and fine cytoplasmic speckle	Unknown 72 kDa band
R24100	58	NSIP	Negative	Negative
R25095	68	UIP	1/2560 homogeneous	Unknown 25 kDa band
R23038	62	UIP	Negative with strong cytoplasmic speckle	Anti-PL-12
R24526	59	Indeterminate	Negative with cytoplasmic speckle	Negative
R19338	65	UIP	1/640 homogeneous and fine cytoplasmic speckle	Unknown 72 kDa band
R25133	60	NSIP	Negative	Unknown 30 kDa band
R26806	36	UIP	Negative with strong cytoplasmic speckle	Anti-PL-12
R26812	51	UIP	Negative with strong cytoplasmic speckle	Anti-PL-12
R26518	64	NSIP	Homogeneous 1/640	Anti-Topoisomerase
R26895	58	UIP	Negative with weak fine cytoplasmic speckle	Unknown 31, 32 and 145 kDa bands
R15264	75	UIP	Negative with strong cytoplasmic speckle	Unknown 62-63 kDa band (Ha antigen?) Possible synthetase (not confirmed)

Table 22: Clinical features of patients with presumed idiopathic interstitial pneumonia with recognised CTD-autoantibodies tested by IPP

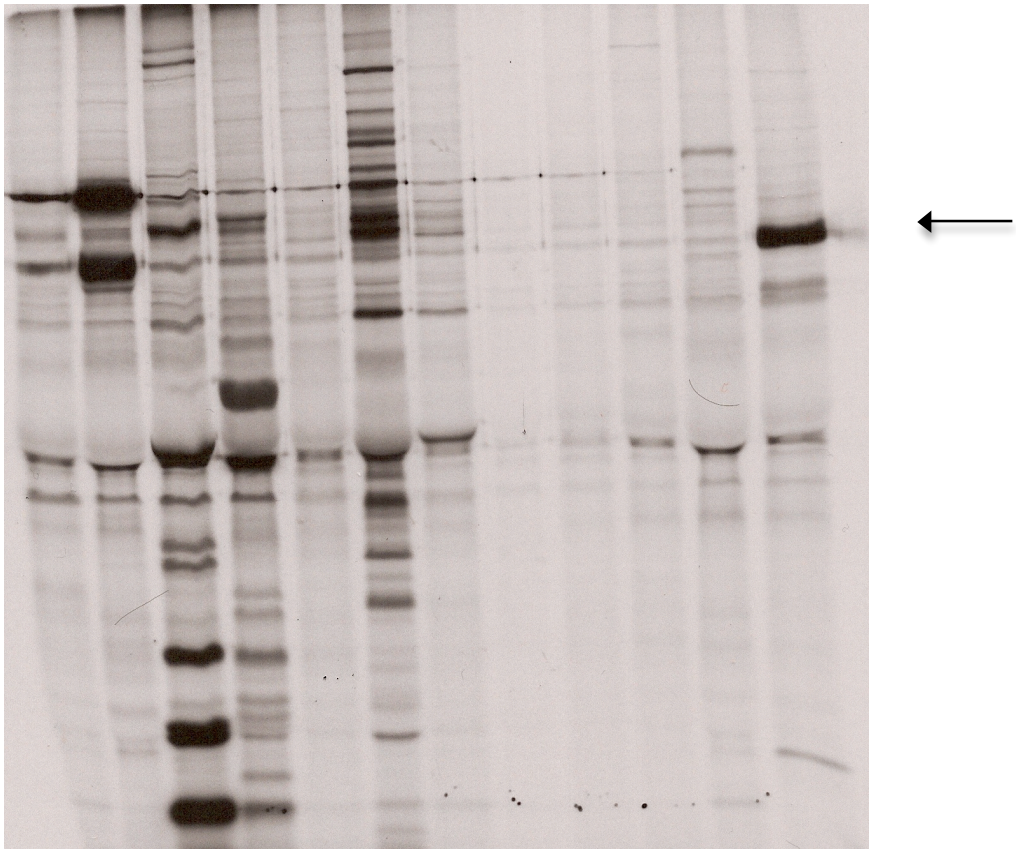
	Clinical Feature					
Patient	RP	MH	Fever	Arthralgia	Subtype of IP	Autoantibody
R26806	Yes	Yes		Yes	UIP	Anti-PL-12
R26518	Yes				NSIP	Anti-Topo
R15264	Yes	Yes	Yes		UIP	Anti-p62 (Anti-Ha?)
R24101		Yes			NSIP	Anti-PL-7
R23038			Yes	Yes	NSIP	Anti-Jo-1
R26812				Yes	UIP	Anti-PL-12

RP: Raynaud's phenomenon
MH: Mechanic's hands
Topo: Topoisomerase

Figure 21: Immunoprecipitation study in idiopathic lung disease patients

21A

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract using normal serum (lane 1), reference anti-PL-12 and reference anti-PL-7 sera (lane 2), reference anti-Jo-1, reference anti-U1-RNP and reference anti-RNAP-II sera (lane 3), reference anti-PM-Scl, reference anti-Ro and reference anti-La sera (lane 4), R23840 serum (lane 5), R23839 serum (lane 6), R23838 (anti-Jo-1) serum (lane 7), R23837 serum (lane 8), R23843 serum (lane 9), R23842 serum (lane 10), R24103 serum (lane 11), and R24101 (anti-PL-7) serum (lane 12 – arrow).

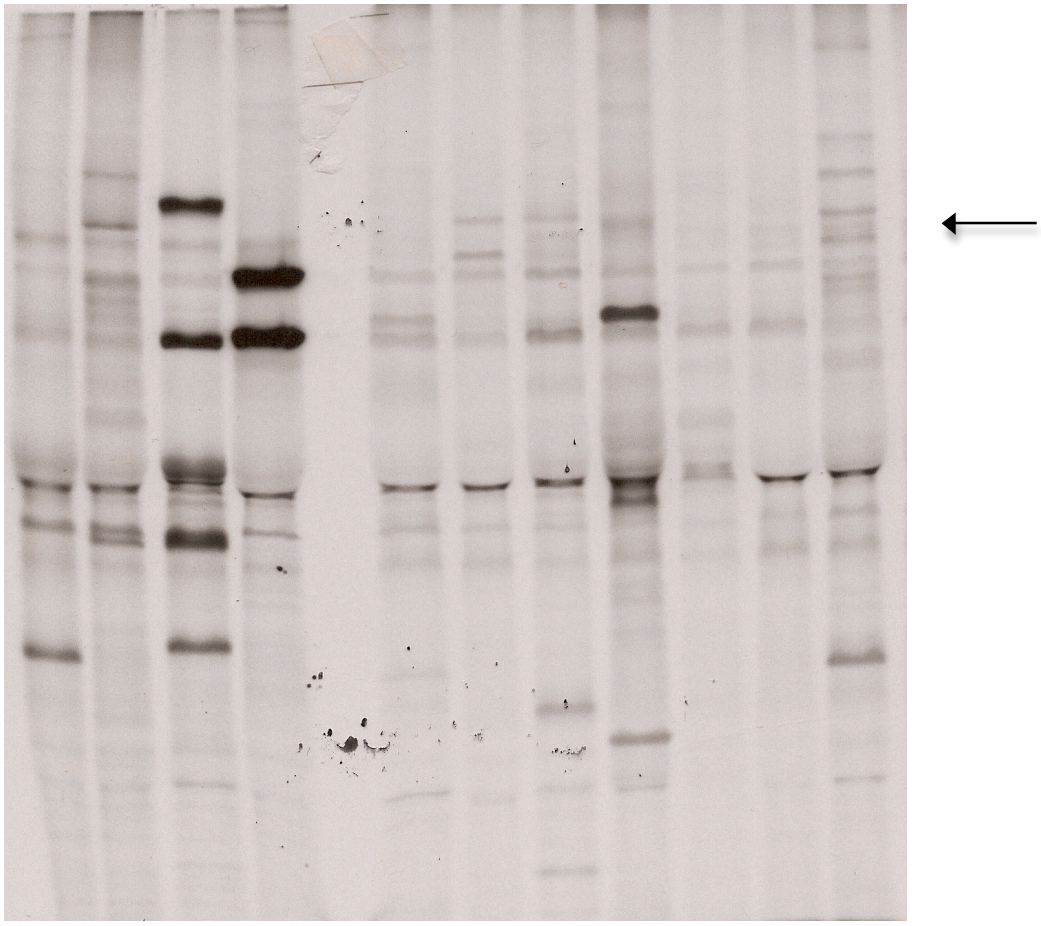


1 2 3 4 5 6 7 8 9 10 11 12

Figure 21: Immunoprecipitation study in idiopathic lung disease patients

21B

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract using reference anti-U3-RNP serum (lane 1), R2518 serum (anti-Topoisomerase) (lane 2), reference anti-mitochondrial serum (lane 3), reference anti-Ku serum (lane 4), R24102 serum (lane 5), R24100 serum (lane 6), R19803 serum (lane 7), R19338 serum (lane 8), control normal serum (lane 9), R24526 serum (lane 10), and R25133 serum (lane 11). Arrow – 100 kDa.

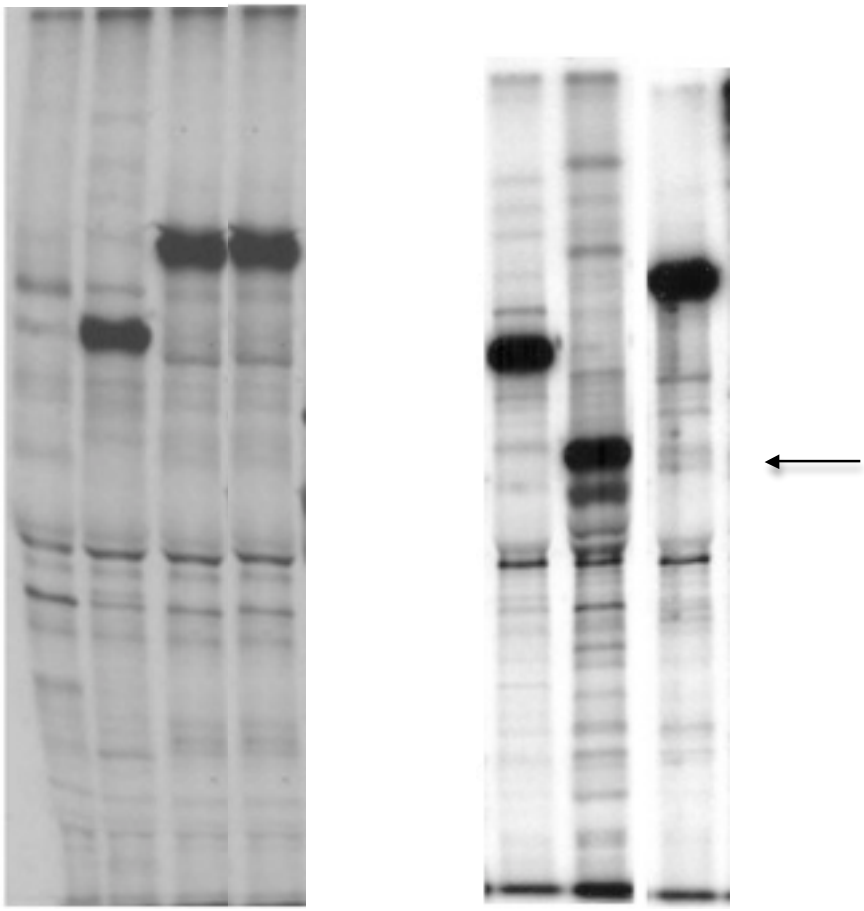


1 2 3 4 5 6 7 8 9 10 11

Figure 21: Immunoprecipitation study in idiopathic lung disease patients

21C

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract using control normal serum (lane 1), reference anti-PL-7 serum (lane 2), R26806 serum (anti-PL-12) (lane 3), R26812 serum (anti-PL-12) (lane 4), reference anti-PL-7 serum (lane 5), R15264 (anti-p62 / Ha) serum (lane 6) (arrow), and reference anti-PL-12 serum (lane 7).



7.1.2 Anti-p140 autoantibodies in adult IIM

Introduction

As described in Chapter 6, a major autoimmune target in JDM is the p140 autoantigen, likely to be the nuclear matrix protein NXP-2. Following detailed serological analysis of adult IIM patients recruited to the RNHRD cohort study and AOMIC UK Registry (work described in Chapters 3 and 4), a subset were noted to also immunoprecipitate a p140 kDa band. Therefore, preliminary work was undertaken to investigate if autoantibodies to the same p140 autoantigen, are present in adult myositis sera.

Patients and Methods

Clinical data and serum samples were available from the UK Adult Onset Myositis Immunogenetic Collaboration (AOMIC) and the RNHRD IIM cohort study (as described in Chapters 2-4). As previously described, serum samples were autoantibody typed by immunoprecipitation using [^{35}S] labelled K562 cells. Immunofluorescence using HEp-2 cells and fluorescein-labelled anti-human immunoglobulin was completed on adult anti-p140 positive samples.

Results

Sera from 266 myositis patients were screened for anti-p140 autoantibodies. Overall, seven cases (3%) were positive for anti-p140 autoantibodies, which were detected exclusively in 5% of DM patients. From the RNHRD cohort study, two reference sera (R18883 and R23389) were positive for autoantibodies to a p140 kDa band. A further five patients' sera were found to recognise a 140 kDa band on SDS-PAGE. No anti-p140 antibody positive patients were positive for other recognized autoantibodies (see Figure 22). IPP experiments using cell extract pre-depleted with reference adult-p140-positive sera (R18883) suggest that the identity of the p140 target in adult DM may be the same as the p140 protein recognised in JDM, and different to the p155/140 autoantigen (see Figure 23). ANA IIF results are shown in Table 23. The major clinical features of anti-p140-positive patients were heliotrope rash (73%), Gottron's lesions (82%), periungal erythema (91%) and systemic involvement including weight loss or fever (78%). In particular, five out of seven (71%) anti-p140-positive adult patients had interstitial pneumonia. There was no cancer-associated myositis in the adult anti-p140 positive subset. In contrast to anti-p140-positive JDM patients where calcinosis is a significant feature, this was only present in one patient.

Dr Zoe Betteridge (and HG) have since conducted further work. This work has been published in abstract form:

Betteridge ZE, Gunawardena H, Chinoy H, Vencovsky J, Allard S, Gordon PA, Cooper RG, McHugh NJ. Autoantibodies to the p140 autoantigen NXP-2 in adult dermatomyositis. Arthritis Rheum 2009;60:S815.

In total, serum and clinical data has now been analysed in 443 adults with myositis (PM and DM), based on the Bohan and Peter diagnostic criteria, recruited to the Adult Onset Myositis Immunogenetic Collaboration, UK, RNHRD Cohort and the Institute of Rheumatology, Prague, CZ. All sera were autoantibody typed by immunoprecipitation using ^{35}S-labelled K562 cells. Immunofluorescence using Hep-2 cells and fluorescein-labelled anti-human immunoglobulin was completed on p140 positive samples.

Thirteen (2.93%) adult patients with IIM were positive for anti-p140 autoantibodies. All anti-p140 positive patients had DM. The prevalence of anti-p140 autoantibodies specifically in the DM cohort was 5.86%. Anti-p140 autoantibodies were not detected in sera from normal healthy controls or PM, myositis-overlap, SSc or SLE patients.

The major clinical associations of anti-p140 autoantibodies in adults are shown in Table 24. In particular, the frequency of interstitial pneumonia in anti-p140 positive patients was 61.5% in comparison to 26.2% in the DM anti-p140 negative group ($p=0.016$). In addition, no p140 positive patients had cancer-associated myositis and in comparison with the JDM cohort, where calcinosis was a significant association, calcinosis was only present in two adult p140-positive patients (15.4%).

Discussion

Anti-p140 autoantibodies form a further serological subset in adult DM. In addition, preliminary data suggests that the clinical associations of anti-p140 autoantibodies in adults differ from JDM. In particular, lung disease appears to be a major feature of anti-p140-positive adult DM associated with hallmark cutaneous disease. Screening of larger IIM cohorts will further characterise the anti-p140 clinical phenotype in comparison to other IIM serological subsets, specifically novel specificities anti-SAE and anti-p155/140.

Figure 22: Immunoprecipitation of anti-p140 autoantibodies in adult DM

Autoradiogram of 10% SDS-PAGE of immunoprecipitates of [^{35}S] labelled K562 cell extract using normal serum (lane 1), reference anti-p155/140-adult DM serum (lane 2), R18883 anti-p140-positive serum (lane 3), AOMIC M203 anti-p140-positive serum (lane 4), AOMIC M238 serum – unknown p140-142 doublet (lane 5), AOMIC M260 serum – faint band at 142 kDa (lane 6), AOMIC M352 serum – negative (lane 7), AOMIC M86 serum – weak unknown p140-142 doublet (lane 8), AOMIC M89 anti-p140-positive serum (lane 9), AOMIC M136 anti-p140-positive serum (lane 10), AOMIC M145 anti-p140-positive serum (lane 11), AOMIC M212 anti-p140-positive serum (lane 12), AOMIC M347 serum – negative (lane 13), and R23389 anti-p140-positive serum (lane 14). Arrow indicates 140 kDa band.

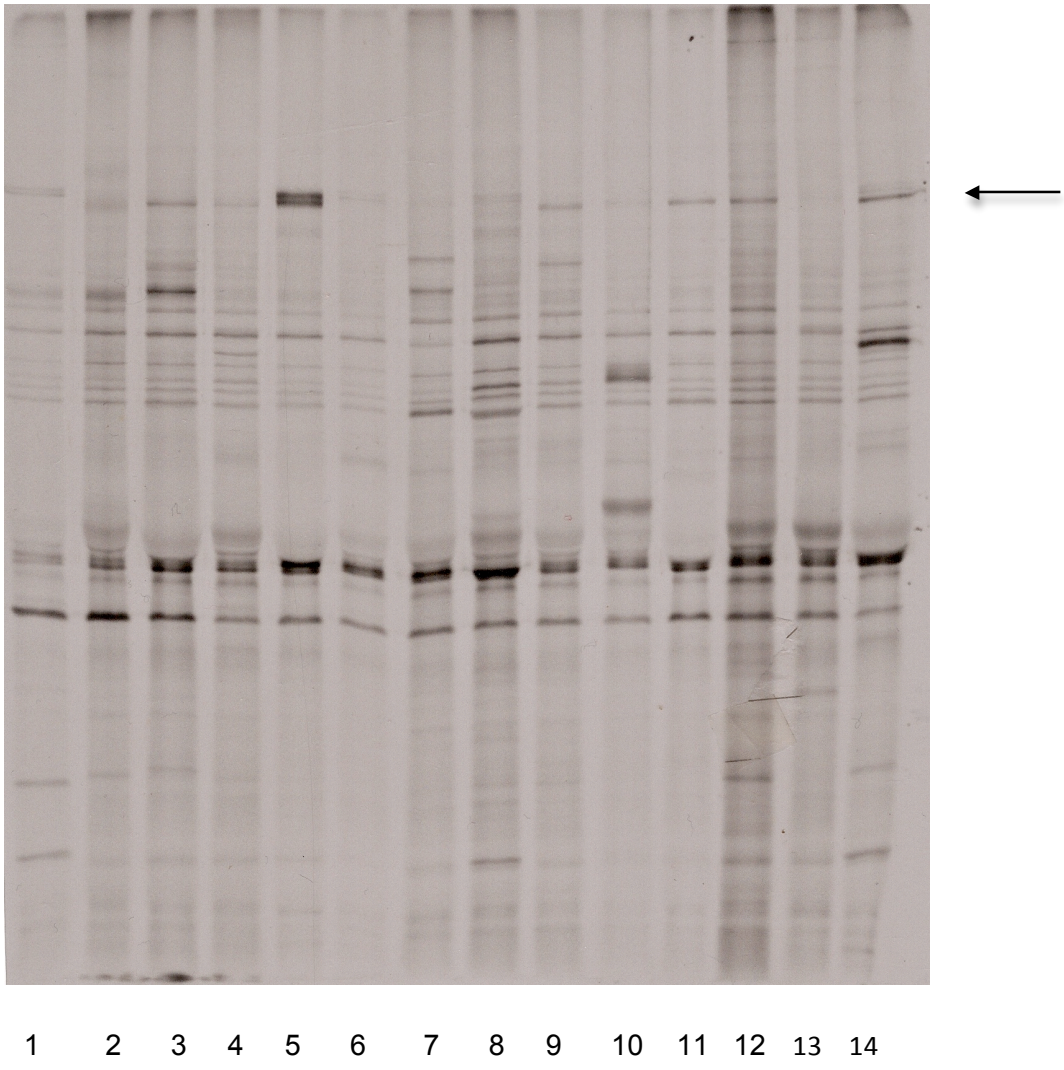
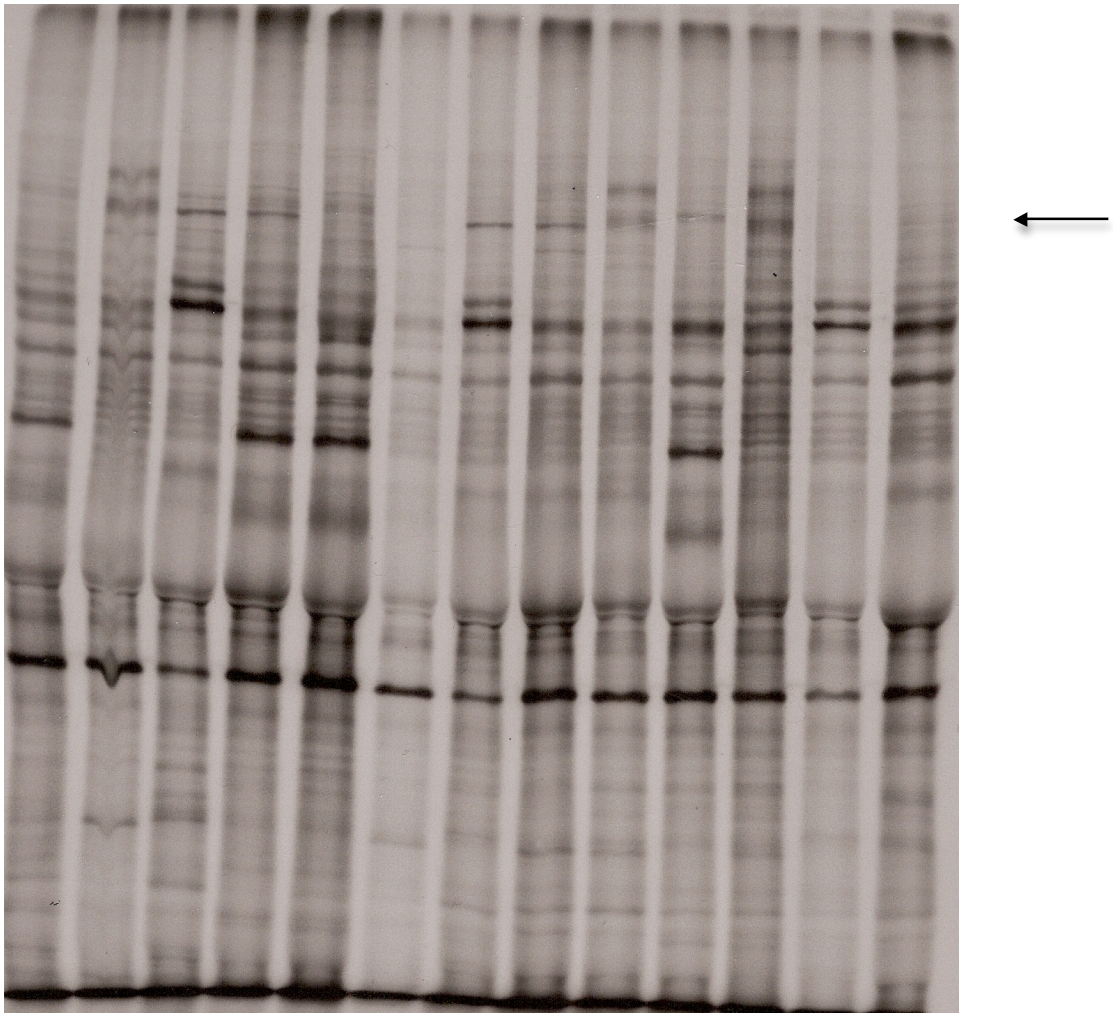


Figure 23: Immunodepletion experiments with reference anti-p140-positive JDM and adult DM sera, and anti-p155/140-positive JDM sera

Autoradiogram of 10% SDS-PAGE of immunoprecipitates using anti-p140-positive JDM serum with p140 predepleted [^{35}S] labelled cell extract (using reference R18883 anti-p140-positive adult serum) (see corresponding negative lane 13) / using R23389 anti-p140-positive adult DM serum with p140 predepleted ^{35}S–labelled cell extract (using reference R18883 anti-p140-positive adult serum) (see corresponding negative lane 12) / using reference anti-p155/140-positive adult DM serum with p140 predepleted [^{35}S] labelled cell extract (using reference R18883 anti-p140-positive adult serum) (see corresponding p155/140 bands lane 11). Arrow indicates 140 kDa band.



1 2 3 4 5 6 7 8 9 10 11 12 13

Table 23: Anti-p140 autoantibody ANA IIF patterns (adult DM)

Sample	ANA IIF pattern
R18883	Fine speckle nucleolar sparing and fine cytoplasmic speckle
R23389	Fine speckle nucleolar sparing 1/40
M89	Homogeneous
M136	Fine speckle nucleolar sparing
M145	Homogeneous
M203	Fine speckle nucleolar sparing 1/40
M212	Homogeneous

Table 24: Clinical Features of anti-p140 autoantibodies in adult dermatomyositis

	Anti-p140 autoantibodies	
Clinical Feature	Positive (n=13)	Negative (n=430)
Gender	53.8% Female	74.2% Female
Gottron's Lesions	92.3%	74.3%
Heliotrope Rash	76.9%	66.8%
Raised Creatinine Kinase	53.9%	76.4%
Systemic Features	76.9%	53.4%
Cancer	0%	14.9%
Interstitial pneumonia	61.5% *	26.2%
Weakness	69.2%	88.3%

*P_{uncorr}=0.016 (OR 4.5 95% CI: 1.4-14.4)

7.2 Summary and Final Discussion

7.2.1 Myositis-specific autoantibodies

The work described in this thesis combined with various studies over the past few years provides evidence highlighting the importance of autoimmunity in myositis. Up to 80% adult patients and 60% of juvenile patients now have well defined MSAs and MAAs when tested by detailed serological techniques.

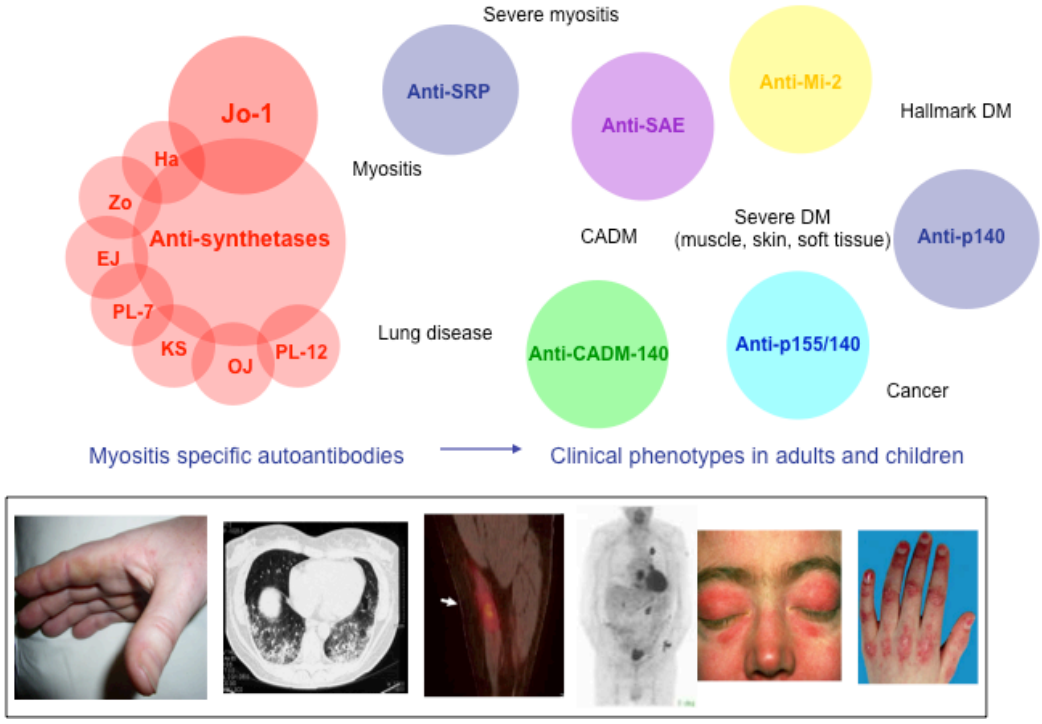
- Eight anti-synthetase autoantibodies collectively define the anti-synthetase syndrome, characterised by myositis, skin disease including mechanic's hands and Gottron's papules, non-erosive arthritis, fever, Raynaud's and interstitial pneumonia. Lung involvement is a major complication and may the predominant clinical manifestation.
- Anti-SRP autoantibodies define patients severe acute / subacute necrotising myopathy, which may be refractory to standard treatment.
- Anti-Mi-2 autoantibodies define patients with classic hallmark DM who appear to respond well to standard treatments and run a monocyclic disease course.
- Anti-CADM-140 (MDA5) autoantibodies appear to be unique to Asian clinically-amyopathic DM patients with a high risk of progressive acute interstitial pneumonia.
- Anti-p155/140 is a novel autoantibody found in both adult DM and JDM. This specificity defines patients with severe cutaneous disease, and is associated with cancer-associated myositis in older adults.
- Anti-SAE autoantibodies are detected in adult DM who may present with clinically-amyopathic DM first, progressing to myositis and systemic features. Interstitial lung involvement appears infrequent.
- Anti-p140 autoantibodies are a major serological subset in JDM, and are associated with calcinosis.

The identification and characterisation of an increasing number of MSAs and their corresponding targets has emphasised how we should now consider a clinico-serological classification in IIM, rather than simply diagnose patients with PM, DM or CTD-myositis overlap. In the future, if MSAs are used to define patients into clinical syndromes this may help predict outcomes and thus influence treatment strategies.

Table 25: Summary: Myositis-specific autoantibodies, autoantigen targets and clinical features

Autoantibodies	Target autoantigen and function	Clinical Phenotype	Autoantibody frequency (%)	
			Adults	JDM
Anti-ARS Anti-Jo-1 Anti-PL-7 Anti-PL-12 Anti-EJ Anti-OJ Anti-KS Anti-Ha Anti-Zo	ARS – intracytoplasmic protein synthesis Histidyl Threonyl Alanyl Glycyl Isoleucyl Asparaginyl Tyrosyl Phenylalanyl	Anti-synthetase syndrome Myositis, mechanic's hands, Gottron's papules, arthritis, fever, Raynaud's phenomenon, high frequency of interstitial pneumonia	30-40%	1-3%
Anti-SRP	SRP – intracytoplasmic protein translocation (6 polypeptides and ribonucleoprotein 7SLRNA)	Acute onset necrotizing myopathy (severe weakness, high CK) May be refractory to treatment	5%	<1%
Anti-Mi-2	Helicase protein – nuclear transcription (Forms the NuRD complex)	Adult DM and JDM (hallmark cutaneous disease, milder muscle disease with good response to treatment)	<10%	<10%
Anti-p155/140	TIF1-γ (p155) – nuclear transcription + cellular differentiation	CAM in adult DM Severe cutaneous disease in adult DM and JDM	13-21%	23-29%
Anti-p140	Likely to be NXP-2 – nuclear transcription + RNA metabolism	JDM with calcinosis	N/A	23%
Anti-SAE	SAE - post-translational modification (targets include transcription factors)	Adult DM May present with CADM first	5%	N/A
Anti-CADM-140	Intracytoplasmic MDA5 – innate immune responses against viral infections	CADM Rapidly progressive interstitial pneumonia	Overall - unknown	N/A

Figure 24: Summary: Myositis-specific autoantibodies and clinical features



7.2.2 Pathogenic mechanisms: MSAs and autoantigens as clues

There is strong evidence that autoimmune mechanisms play an important role in both polymyositis (PM) and dermatomyositis (DM). Previous studies have reported that around 40-50% of adults and 20-30% of juvenile IIM patients have the presence of high-titre autoantibodies. Myositis autoantibodies can be categorised into myositis associated autoantibodies (MAAs) and myositis specific autoantibodies (MSAs). The MAAs; anti-U1-RNP, anti-U3-RNP (fibrillarin), anti-PM-Scl and anti-Ku are principally seen in myositis-scleroderma overlap syndromes (240), whereas the MSAs are highly selective, mutually exclusive and are associated with particular genotypes and clinical phenotypes within the myositis spectrum (6, 8, 9, 241). This thesis describes a comprehensive, clinical and serological study of adult and juvenile idiopathic inflammatory myopathy. Based on this body of work combined with additional preliminary studies, the frequency of MSAs/MAAs is nearer 80% in adults and 60% in children, therefore dispelling the notion that the majority of myositis cases are 'seronegative' (241-243).

MSAs are directed against both cytoplasmic and nuclear components of the cell involved in key regulatory intra-cellular processes, including gene transcription, protein translocation and anti-viral responses. Interestingly, autoantigens with analogous cellular functions are associated with similar clinical subsets. For example, as described in previous chapters, the anti-ARS autoantibodies target functionally related cytoplasmic tRNA enzymes involved in protein synthesis. This group of autoantigens form the largest subset recognised in adult IIM patients, and are associated with distinct clinical features collectively termed the anti-synthetase syndrome. A further subset of myositis patients with severe necrotising myopathy is characterized by the presence of autoantibodies directed against the signal recognition particle (SRP) (121, 122). SRP is a cytoplasmic ribonucleoprotein complex that recognizes secreted and membrane-bound proteins regulating protein translocation through the endoplasmic reticulum.

In contrast, autoantibodies associated with DM clinical phenotypes, such as anti-Mi-2 and novel MSAs anti-p155/140 and anti-p140, target nuclear proteins (126, 143, 157, 158, 161). Mi-2 is a nuclear helicase protein that forms part of the nucleosome remodelling deacetylase (NuRD) complex playing a role in gene transcription, specifically chromatin remodelling (129). Targoff *et al* has identified the p155 target of the p155/140 complex as transcriptional intermediary factor 1-gamma (TIF1-γ), a nuclear protein involved in cellular differentiation (159). In addition, the p140 target

has been identified as nuclear matrix protein NXP-2, which plays a role in RNA metabolism and maintenance of nuclear architecture (228, 234). Finally, anti-SAE autoantibodies target the small ubiquitin-like modifier activating enzyme subunits involved in post-translational modification that is located in both the nucleus, but unlike other DM-associated autoantigens, it is also found in the cytoplasm of cells (168). The potential relationship between the DM-specific autoantigens is thus of interest. It is possible SAE plays a central role forming stable conjugates with other proteins including transcription factors (168). Interestingly, a specific target of sumoylation is a protein (p66) that has been shown to mediate transcriptional repression of the Mi-2/NuRD complex (178). Furthermore, NXP-2 has been reported to have a role in sumoylation-mediated regulation of transcription (235). Post-translational modification can lead to the generation of self-antigens and so this observation suggests shared pathogenic mechanisms.

The exception to the above observations may be the anti-CADM-140 autoantibody, now described in two Asian DM cohorts with CADM and severe lung involvement (130, 131, 244). The identity of this cytoplasmic protein CADM-140 has recently been discovered to be melanoma-differentiation-associated gene 5 (MDA5) (131, 244). The MDA5 autoantigen appears to be distinct in terms of its biological function and disease expression. MDA5 is involved in the innate immune defence against viral infections through the detection of viral dsDNA (132). This observation may be explained by the fact that anti-CADM-140-autoantibody DM appears to be a unique clinical subset found only in Asian ethnicity, with specific genetic and environmental determinants.

In general, the systemic autoimmune diseases are a genetically complex heterogeneous group of diseases in which the immune system attacks diverse but highly specific intracellular self-structures. In IIM, the striking association between specific autoantigen targets and clinical phenotypes, to the degree that IIM can now be classified into more homogeneous subsets, suggests that the autoantigen itself may be central in determining disease expression. Moreover, in IIM there are key target tissues, in particular the muscle, the skin, the lungs, and in some cases tumour tissue. It is possible that in IIM syndromes, disease initiation is dependent on the structure and concentration of autoantigens (i.e. novel non-tolerized forms – truly novel epitopes or previously recognised but modified epitopes that lower the threshold for autoreactive T cells to be activated) in certain localised pro-immune microenvironments. Several studies have provided insight not only into the potential

importance of the autoantigen target but also it's expression in specific microenvironments. Two studies have highlighted how certain autoantigenic -tRNA synthetases (histidyl, asparaginyl and tyrosyl) have chemoattractant properties and can induce leukocyte migration via the CCR5 and CCR3 receptors respectively (135, 245). In comparison, non-antigenic -tRNA synthetases (aspartyl and lysyl) do not activate chemokine receptors. In addition, mononuclear cells expressing chemokine receptors are present in myositis muscle but not normal muscle (135). The authors suggested that autoantigenic ARS are over-expressed in damaged muscle cells and their pro-inflammatory properties promote the immune response, which leads to the development of myositis.

Several studies have now demonstrated that certain autoantigens are enriched in lesional tissue involved in IIM. In the seminal paper by Casciola-Rosen *et al* several myositis-specific and associated autoantigens (including the Jo-1 and Mi-2 proteins were shown to be upregulated in myositis muscle in comparison to normal muscle especially in regenerating muscle cells, rather than mature myotubes (107). Zampieri *et al* demonstrated similar findings in newborn skeletal muscle, in the absence of infiltrating inflammatory cells. Using immunocolocalisation techniques, anti-Jo-1 sera showed a positive reaction within the cytoplasm of tibialis anterior muscles (TA) from newborn rat cyrosections and anti-Mi-2 positive serum gave a homogenous staining of myonuclei. In contrast, sections of TA from adult rats gave negative staining with both anti-Jo-1 and anti-Mi-2 sera. In addition, staining of Jo-1 and Mi-2 paralleled the staining with anti-MHCemb, further indicating that expression of Jo-1 and Mi-2 is enhanced in myotubes (246). Collectively, these findings support the hypothesis that the presence of candidate myositis autoantigens during reparative myogenesis can drive induction and propagation of the autoimmune response.

It has been proposed that susceptibility to the serine protease, granzyme B, is strongly predictive of autoantigen status. Casciola-Rosen *et al* have shown that isoleucyl-, histidyl- and alanyl-tRNA synthetases, Mi-2 and SRP72 are all cleaved by granzyme B, leading to the release of unique fragments that contain autoantigenic epitopes (104). Work by Levine *et al*, has emphasized the potential pathogenic role of proteolytic cleavage of autoantigens in lesional tissues. They demonstrated that the histidyl-tRNA protein exists in two conformations and whilst there are similar overall expression levels of Jo-1 in different tissues, the granzyme B cleavable form is enriched in the alveolar epithelial layer of lung (108). Therefore, the theory that

distinct microenvironments may shape disease expression was emphasised with this finding that a novel conformation of Jo-1 is enriched in the lung in comparison to other tissues including muscle. This has lead to the suggestion that the initiating target tissue for the autoimmune response in the anti-Jo-1 syndrome is the lung with secondary attack to muscle.

Further evidence implicating autoantigen driven responses was suggested in a study by Katsumata *et al* (133). They generated an antigen-induced model of IIM following immunisation of congenic mice with either human or murine Jo-1 protein or amino terminal peptides of Jo-1 protein. Early antibody responses demonstrated strong species-specificity with limited cross recognition of Jo-1 between species. Studies on serum samples 8 weeks after immunisation revealed epitope spreading with the breakdown of B-cell tolerance to ubiquitously expressed native target autoantigens. The presence of anti-Jo-1 IgG isotypes indicated class switching and T-cell dependant B-cell responses, likely reflecting a breakdown in T-cell tolerance to self Jo-1. Assessments of mice after murine Jo-1 immunization demonstrated the presence of skeletal muscle inflammation and interstitial lung disease, which are clinical characteristics of the ASS clinical phenotype in humans.

Work on the Mi-2 protein gives further emphasis to the potential role of target proteins in disease expression. Casciola-Rosen *et al* have previously demonstrated that Mi-2 is preferentially expressed in DM muscle rather than PM muscle, supporting the association between DM and Mi-2 (107). Using a conditional gene targeting approach in a mouse model, Kashiwagi *et al* demonstrated that Mi-2 is essential for the development and repair of the basal epidermis (134). Furthermore, studies by Burd *et al* have reported that following UV radiation, Mi-2 protein levels in a human keratinocyte cell line are rapidly up-regulated and maintained by an increase in translation efficiency through a regulatory element in the 5'-UTR region of mRNA and increased protein stability. This suggests enhanced expression of Mi-2 in UV exposed keratinocytes leads to the initiation of autoimmunity and subsequently the DM phenotype associated with classic skin lesions. Autoantibodies to other members of the NURD complex have not been reported in IIM, and the effects of UV radiation do not significantly affect other NURD proteins, leading to the hypothesis that Mi-2 may act independently of the NURD complex in response to DNA damage (247).

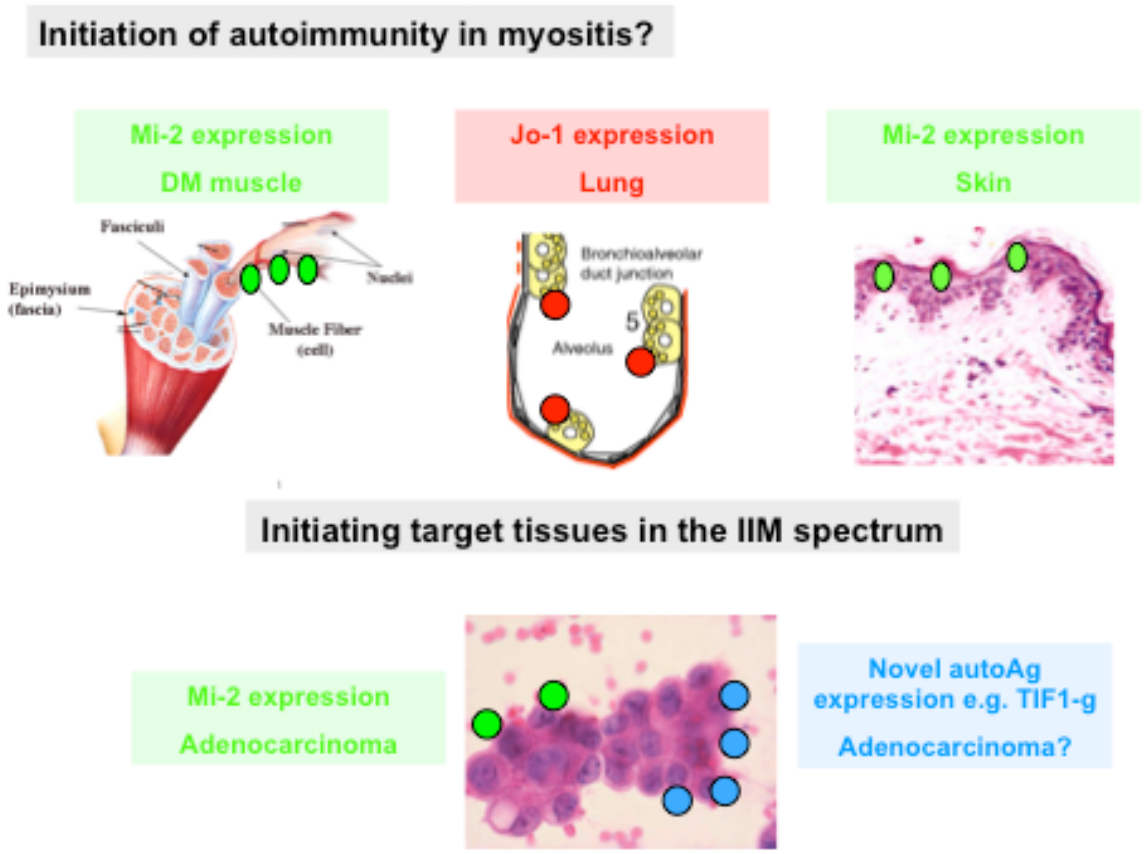
The association between malignancy and myositis, particularly in adult DM is well recognised. Casciola-Rosen *et al* have demonstrated that Mi-2 and Jo-1 expression is increased in certain tumours including breast and lung adenocarcinomas (107). Okada *et al* also demonstrated the presence of autoantibodies to the DNA mismatch repair protein PMS1 (248), a further myositis autoantigen in patients with pancreatic adenocarcinoma, with protein levels increased in the corresponding lesional tissue (249). Furthermore, it has been demonstrated that tumour cells express low levels of specific effector caspases (250, 251), leading to the suggestion that with certain forms of cancer there may be a relative increase in other proteolytic enzymes, including granzyme B. It is therefore plausible that there may be generation of uniquely cleaved autoimmune fragments in carcinoma cells. Suber *et al* have suggested a hypothesis that in certain circumstances, the autoimmune response in myositis is secondary to a primary anti-cancer response. A further trigger, such as a muscle-trophic virus then leads to enhanced expression of autoantigens promoting activation of autoreactive lymphocytes and tissue damage (138). This theoretical model is supported by Mimuro *et al*, who demonstrated that the cancer-associated anti-p53 antibody is also observed in PM and DM patients, even when not associated with malignancy (252). The discovery of the p155/140 autoantigen and its clear association with cancer in adult DM is an exciting breakthrough, and may increase our understanding of the relationship between autoimmunity and cancer (143, 157, 158). What is fascinating is the fact that the same protein is an autoimmune target in JDM, which does not appear to be associated with malignancy (see Chapter 6) (161), and this also appears to be the case in younger adults with amyopathic DM (as described in Chapter 4). Perhaps some perturbation of p155/140 in proliferating cells combined with a more efficient anti-cancer response by a younger immune system may be the answer. Interestingly p155 (TIF1-γ) has been shown to inactivate Smad-4, which regulates TGF-β signalling, thus promoting cell growth and differentiation (including malignant tumours) (253). Further studies to investigate p155/140 autoantigen expression and conformation in cancer tissue and compare this to levels in other lesional tissues may be revealing. Whilst the anti-p155/140 autoantibody is the only MSA significantly associated with cancer there is evidence for roles of other autoantigen targets in malignant processes. These include autoantigenic members of the aminoacyl tRNA synthetase family; tyrosyl-, isoleucyl-, phenylalanyl- and glycyl-tRNA synthetase. Of particular interest is the preferential expression of the interstitial lung disease associated autoantigen, phenylalanyl-tRNA synthetase (alpha-subunit) in solid lung tumours and acute myeloid leukaemia (254-260). In addition, MORC3 (NXP-2 / p140) is essential for

regulating the sub-nuclear localisation of p53 (261), and MDA5 activates IkB (IKK) related kinases, known to have a role linking chronic inflammation and cancer (262). The next question of whether MSAs are simply epiphenomena or directly linked to pathogenesis remains uncertain. Two recent publications have highlighted the potential role of anti-Jo-1 autoantibodies in disease activity and pathogenesis. Stone *et al* investigated the association between anti-Jo-1 levels and myositis disease activity (263). The authors performed a cross-sectional study of 81 anti-Jo-1 positive patients and disease activity was assessed using CK levels and the myositis disease activity assessment tool. Anti-Jo-1 levels were measured using a commercial ELISA and a custom ELISA using recombinant human autoantigen. Anti-Jo-1 levels showed modest correlation with CK, myositis and joint disease activity. Longitudinal data was available on 11 patients that showed serial anti-Jo-1 levels did correlate with CK and disease visual analogue scales. In the second study, Eloranta *et al* concluded that immune complexes containing either anti-Jo-1 or anti-Ro in the presence of RNA may act as endogenous inducers of type 1 interferon alpha (IFN-α) (80). Together, these findings suggest that anti-Jo-1 autoantibodies may play a role in disease propagation, and it would be of interest to see whether other MSAs correlate with disease activity and are able to induce pro-inflammatory cytokine activity. Therefore in IIM, disease propagation and/or disease flares may be autoantibody driven in an already primed pro-immune environment, which leads to autoantibody mediated autoantigen capture, presentation and opsonisation.

It now appears clear that in IIM specifically targeted autoantigens share unique properties, are preferentially expressed in disease-associated tissues and have a role in disease initiation and propagation. Investigating the structure and function of target molecules, and whether autoantibodies themselves have functional roles appears critical for understanding the pathogenic mechanisms in this complex spectrum of diseases. This in turn, may lead to therapeutic advances including the development of more targeted treatments.

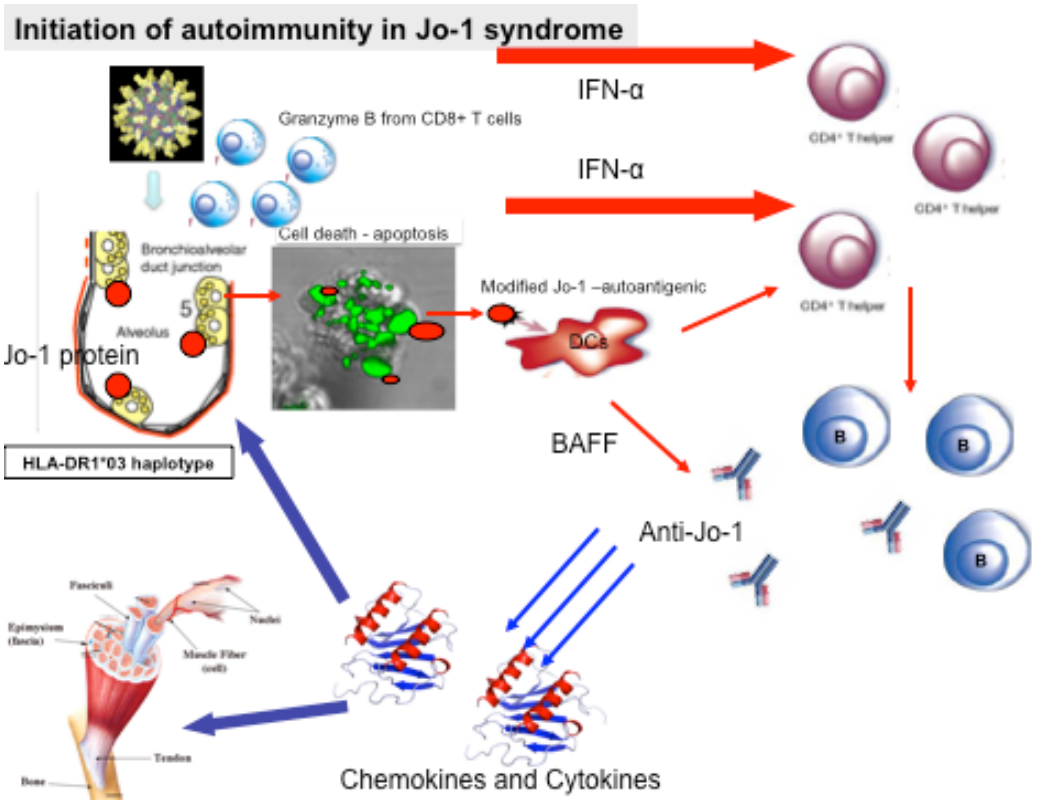
7.2.3 Hypothetical models of autoreactivity and pathogenesis in myositis subsets

Figure 25: Summary of observations: autoantigens as clues in target tissues



- The Mi-2 protein in enriched in DM muscle in comparison to PM muscle and normal muscle (107).
- In mouse models, the Mi-2 protein plays a critical role in the development of the skin basal epidermal layer (134).
- In vitro, UV light upregulates Mi-2 expression in human keratinocyte cell lines (264).
- The Mi-2 protein is enriched in adenocarcinoma tissue (107).
- Expression of the Jo-1 protein is highest in the lung in comparison to other tissues including muscle (108).
- The Jo-1 protein is highly susceptible to granzyme B cleavage in the lung and the immunogenic form of Jo-1 in vivo exists unbound (108).
- Mice immunised with the Jo-1 protein develop the muscle inflammation and lung fibrosis synonymous with the anti-synthetase syndrome (133).

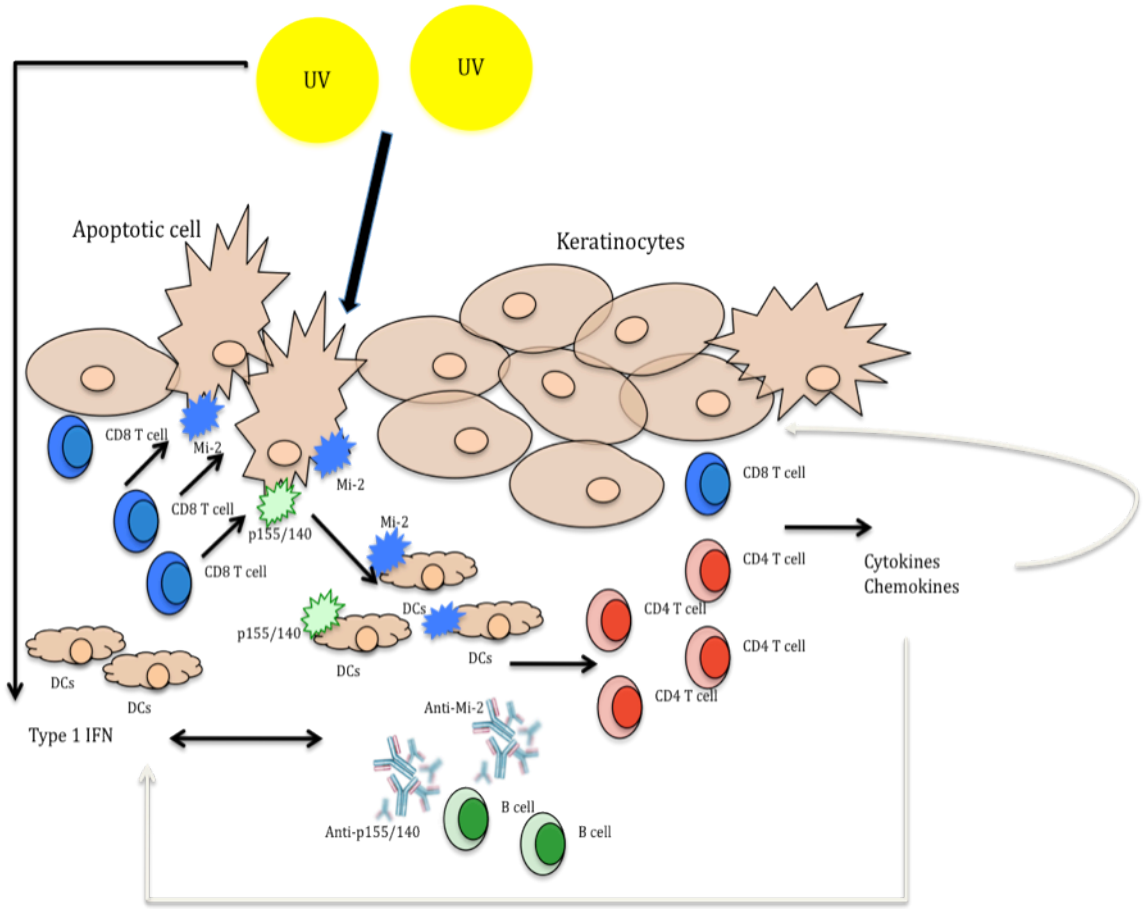
Figure 26: Lung model



This model is based on the observation and works of others (80, 103, 104, 106, 108, 135, 137, 265-267). An environmental trigger such as a lung trophic virus and/or an environmental toxin (e.g. smoking leading to damage of the lung epithelial barrier) may initiate the autoimmune response in genetically susceptible individuals (e.g. HLA-DR3 haplotype). Virus particles incorporate into the alveolar epithelial cells triggering cytotoxic CD8 T cell mediated release of the serine protease granzyme B. This leads to alveolar cell apoptosis and thus modification of the Jo-1 protein via granzyme B cleavage. Granzyme B generated fragments of Jo-1 become immunogenic. Jo-1 is chemo-attractant via CCR5 receptor binding, thus attracting CD4/8 T cells, monocytes and immature dendritic cells (DCs). The Jo-1 autoantigen may be incorporated into DCs and then presented to CD4 T cells, which in turn initiates T cell autoreactivity and autoantibody production by B cells. This mechanism is enhanced by anti-viral type 1 IFN responses leading to DCs maturation and further antigen presentation. Anti-Jo-1 autoantibody production is also promoted through the interaction of mature DCs with autoreactive B cells via BAFF. This scenario is supported by the finding that bronchial-alveolar fluid from NSIP lung (the most common subtype of interstitial pneumonia in Jo-1 myositis) is rich in CD8 T cells. Therefore, the initiating target tissue in Jo-1 ASS may be the lung with muscle and/or skin targeted at later stages of injury as Jo-1 autoantigen

levels and corresponding autoantibody production increases. It is likely that the other ARS proteins, in particular PL-7 and PL-12 share similar immunogenic properties and expression profiles in the lung, particularly because lung damage is the predominant feature of this subtype of anti-synthetase syndrome.

Figure 27: Skin model: Initiation of autoimmunity in Mi-2 dermatomyositis syndrome



In genetically susceptible individuals (e.g. DRB1*07 in Mi-2 syndrome), UV-light triggers cutaneous inflammation. CD8 T cell mediated keratinocyte cell death leads to upregulation of unique immunogenic fragments of the Mi-2 protein. It is possible that the Mi-2 autoantigen also has chemoattractant properties (similar to the Jo-1 autoantigen), thus recruiting T helper cells and DCs. Antigen presentation promotes T cell autoreactivity and anti-Mi-2 autoantibody production by B cells. This process is further enhanced by UV-light induced IFN production leading to DCs maturation and endothelial cell activation. Finally, loss of normal Mi-2 function disrupts basal epidermal cell layer repair mechanisms propagating epidermal damage. Similar to the Jo-1 lung model, attack on muscle may be a secondary downstream effect in

this model. The p155/140 protein or other DM-specific autoantigens such as SAE may be modified by similar mechanisms.

Cancer model: as proposed by Suber *et al* (138)

It is proposed that perhaps in some patients with cancer-associated dermatomyositis the first event in the pathogenic cascade is the initiation of an anti-tumour response. In the early stages of abnormal cell differentiation, the immune system recognises highly expressed tumour antigens (based on current observations these antigens may include Mi-2 and p155/140). This response is either highly effective leading to efficient removal of tumour cells (before any pathological and clinical manifestations) (perhaps this mechanism may explain why p155/140 is a major autoimmune target in JDM) or ineffective (i.e. development of cancer in an 'older' less efficient immune system). This powerful anti-tumour response combined with other stressors e.g. toxins or trophic viruses leads to muscle or cutaneous injury. For example, muscle cells may undergo apoptosis, and those antigens initially targeted by the anti-cancer response, become upregulated in areas of repair and regeneration. This in turn leads to antigen presentation, autoreactive T cell propagation and autoantibody production. Inflammatory cells and cytokine production promote further injury and thus repair, which sustains the autoimmune response against cancer, muscle and/or skin cells. With the discovery of the p155/140 autoantigen, this hypothesis can now be interrogated.

7.3 Future Work

Future work should focus on increasing our understanding the significance of novel protein targets, from how they are expressed at a cellular level to how they classify patients based upon clinical features.

7.3.1 Screening larger cohorts of IIM patients to further define clinico-serological subsets

Further screening is required to establish the prevalence and disease specificity of novel autoantibodies in a European-wide cohort of adult patients with IIM. With the formation of the European Myositis Consortium, cohorts of adult patients with IIM with linked clinical data and serum samples are now available for analysis. This combined with patients recruited to the UK AOMIC Registry and UK JDM cohort study will form the largest collection of adult and juvenile IIM patients (over 1000 patients) with comprehensive clinical data (see chapter 2, appendix, proforma 3 and 4). MSAs are associated with clinical phenotypes and the study of a large cohort of patients will form a major biomarkers resource. Rather than diagnose patients with PM or DM, the work described in this thesis and future studies will lead to defining IIM patients into clinico-serological syndromes, which in the future will aid physicians to predict clinical outcomes and plan specific treatment regimes.

7.3.2 Investigate the persistence and level of anti- p155/140 in JDM in relation to increasing age and clinical course

Around 20% of children with JDM are positive for anti-p155/140 autoantibodies. The same autoantibody specificity is found in adult DM associated with malignancy. Therefore, it will be important to investigate how these autoantibodies fluctuate over disease course (disease activity and response to treatment). Serial samples on children recruited to the UK JDM Cohort Study have been stored during disease course annually will be screened for anti-p155/140 using IPP. Autoantibody levels can be quantified using immunoprecipitation blotting. Briefly, immunoprecipitations will be completed using 40µl serum (p155/140 and control serum), 2mg protein A Sepharose and 5 mM bis-(sulphosuccinimidyl)-suberate cross-linker. Beads will be incubated in 2ml unlabelled K562 cell extract for a total of 2 hr at 4°C. Samples will be resuspended in 80µl SDS sample buffer and are heated. Proteins are fractionated by 10% SDS-PAGE and transferred to nitrocellulose by Western blotting. Blots will be probed with anti-p155/140 positive sera and bands will be detected using an alkaline phosphatase conjugated donkey anti-human IgG antibody and the BCIP/NBT liquid substrate solution. The immunoblots will then be

quantified by densitometry. Equal loading will be confirmed by spiking samples with a pre-defined amount of a lower molecular weight protein. Blots will be stripped and re-probed for this protein. Autoantibody data will be linked to prospective clinical data collected every six months and recorded on the JDRR database.

7.3.3 Antigen expression

Lesional tissue

As described in section 7.2.2, based on the paradigm suggested by Casciola-Rosen and Rosen that in IIM lesional tissue is enriched for certain autoantigens that, under certain circumstances, may drive a dysregulated autoimmune response, further work is required to investigate this hypothesis. IIM is an excellent model to explore this concept further. Muscle, lung, skin and cancer biopsy material is readily accessible tissue that can be studied directly. Tissue expression can be investigated by immunohistochemistry - biopsy specimens (stored at -80^0C) will be cryostat (6-8 um thick), mounted and dried. Slides will be warmed at room temperature, fixed in ice-cold acetone and air-dried. Sections will be washed in PBS and rinsed in TBS-tween. Sections will be blocked and incubated overnight at 4^0C in a humidified atmosphere with commercial monospecific primary antibodies to myositis specific antigens (SRP54 chicken polyclonal ab14072, Mi-2 rabbit polyclonal 06-878, TIF1-γ mouse monoclonal ab33475, SAE1 sheep polyclonal alx210328, SAE2 rabbit polyclonal ap10659, HARS mouse monoclonal ab50835, TARS mouse monoclonal ab50147, ALARS rabbit polyclonal ab50147, NARS rabbit polyclonal ab50144, GARS rabbit polyclonal ab42905, Isoleucyl tRNA-synthetase rabbit polyclonal ab31533, Phenylalanyl tRNA-synthetase alpha H00002193-A01 mouse polyclonal, Phenylalanyl tRNA-synthetase beta H00010056-A01 mouse polyclonal. Sections will be washed and incubated with the appropriate secondary antibodies (anti-mouse/rabbit/sheep/chicken IgG) conjugated with horseradish peroxidase, stained with 1-Step 4-CN (Perbio) and visualised. Negative controls will be performed by omitting or isotype-matching with the primary antibody. Sections will also be probed with FITC and TRITC conjugates as secondary antibodies in order to co-localise autoantigens by double immunofluorescence with appropriate cell types present in lesional tissue. IPP and immunoblotting on cell extracts will be performed to ascertain the specificity of commercially prepared antibodies prior to immunohistochemistry (as already done with commercial anti-SAE1/2 and anti-NXP-2 demonstrating that these antibodies do recognise corresponding antigen targets derived from human cell lines.

Different cell lines

Although the autoantigen targets can be visualised using ^{35}S, for characterisation with mass spectrometry it may be necessary to scale up the quantity of polypeptide immunoprecipitated (e.g. if it is constitutively expressed in low copy number). The initial approach will be to increase the quantity of protein-A-Sepharose beads and source of antigen. However, subsequently, a combination of sub-cellular fractionation and immunoaffinity chromatography may be used to optimise antigen recovery. In addition, other human cell lines may contain different levels of expressed known and novel myositis autoantigens. Differences of expression in these seemingly ubiquitously expressed proteins will provide additional insight into pathogenic mechanisms.

7.3.4 **Development of quantitative solid-phase assays**

The future development of commercial assays that test for MSAs in routine clinical practice is important. To date, certain myositis autoantigens (Jo-1, PL-7, PL-12, Mi-2 and SRP) have been expressed as recombinant proteins for use in commercial ELISAs and line-blots. An important aim is to develop further assays for the novel MSAs including the detection of anti-p155/140 (p155 autoantigen - TIF1-γ (159)), given the potential importance of this autoantibody in the diagnosis of JDM and its association with malignancy in adult-onset DM. Furthermore, the development of custom-based ELISAs will enable the quantitative analysis of MSA levels in serum samples that can be correlated with measures of disease activity, as previously demonstrated with anti-Jo-1 autoantibodies (263). Other autoantigen systems that may also become strategically relevant include anti-SAE, anti-p140 and anti-CADM-140. To develop sensitive immunoblotting techniques and custom based ELISAs preparations will be enriched with the appropriate autoantigens by a combination of cell fractionation, HPLC and affinity purification using monoclonal antibodies. An alternative approach is to extract mRNA from cell lines e.g. K562, A549 cells to develop a cDNA library. This will be screened by either PCR or using a SEREX. Positive sequences will be expressed in a suitable system for the large-scale production of the recombinant peptide. In addition, commercial supplies of the relevant autoantigen can be sourced e.g. TIF1-γ peptide (AbCam 47108) that will be tested for autoantibody recognition by immunoblotting using a panel of appropriate positive and negative controls. A further approach will be the generation of 20-mer overlapping synthetic peptides spanning the known sequence of autoantigens.

BIBLIOGRAPHY (Dr Harsha Gunawardena)

Selected Peer Reviewed Papers

Betteridge ZE, Gunawardena H, McHugh NJ. Pathogenic mechanisms of disease in myositis: autoantigens as clues. Curr Opin Rheumatol 2009;21:604-9.

Chinoy H, Payne D, Poulton KV, Fertig N, Betteridge Z, Gunawardena H, Davidson JE, Oddis CV, McHugh NJ, Wedderburn LR, Ollier WE, Cooper RG; UK Adult Onset Myositis Immunogenetic Collaboration & UK Juvenile Dermatomyositis Research Group. HLA-DPB1 associations differ between DRB1*03 positive anti-Jo-1 and anti-PM-Scl antibody positive idiopathic inflammatory myopathy. Rheumatology 2009;48:1213-7.

Gunawardena H, Wedderburn LR, Chinoy H, Betteridge ZE, North J, Cooper RG, Ramanan AV, Davidson JE, McHugh NJ, for the Juvenile Dermatomyositis Research Group UK. Autoantibodies to a p140-kd protein in juvenile dermatomyositis are associated with calcinosis. Arthritis Rheum 2009;60:1807-14.

Gunawardena H, Betteridge ZE, McHugh NJ. Myositis-specific autoantibodies – clinical and pathogenic significance in disease expression. Rheumatology 2009;48:607-12.

Gunawardena H, Betteridge ZE, McHugh NJ. Newly identified autoantibodies: relationship to idiopathic inflammatory myopathy subsets and pathogenesis. Curr Opin Rheumatol 2008;20:675-80.

Betteridge ZE*, Gunawardena H*, Chinoy H, North J, Ollier WER, Cooper RG, McHugh NJ. Clinical and HLA-class II haplotype associations of autoantibodies to small ubiquitin-like modifier enzyme, a dermatomyositis-specific autoantigen target, in UK adult-onset Caucasian myositis. Ann Rheum Dis 2008 Oct 17. [Epub ahead of print]. (*first author)

Chinoy H, Platt H, Lamb JA, Betteridge ZE, Gunawardena H, Fertig N, Versani H, Davidson J, Oddis CV, McHugh NJ, Wedderburn LR, Ollier WER, Cooper RG. The PTPN22 gene is associated with juvenile and adult UK Caucasian idiopathic inflammatory myopathy independent of the HLA 8.1 haplotype. Arthritis Rheum 2008;58:3247-3254.

Gunawardena H, Wedderburn LR, North J, Betteridge ZE, Dunphy J, Chinoy H, Davidson JE, Cooper RG, McHugh NJ. Clinical associations of anti-p155/140 autoantibodies in juvenile dermatomyositis. Rheumatology 2008;47:324-328.

Gunawardena H, Harris ND, Carmichael C, McHugh NJ. Microvascular responses following digital thermal hyperaemia and iontophoresis measured by laser Doppler imaging in idiopathic inflammatory myopathy. Rheumatology 2007;46:1483-1486.

Gunawardena H, Harris ND, Carmichael C, McHugh NJ. Maximum blood flow and microvascular regulatory responses in systemic sclerosis. Rheumatology 2007;46:1079-1082.

Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Identification of a novel autoantibody in dermatomyositis directed against small ubiquitin-like modifier activating enzyme. Arthritis Rheum 2007;56:3132-3137.

Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Anti-synthetase syndrome: a new autoantibody to phenylalanyl transfer RNA synthetase (anti-Zo) associated with polymyositis and interstitial pneumonia. Rheumatology 2007;46:1005-1008.

Invited Contributions and Selected Case Reports

Gunawardena H, McHugh NJ. Systemic sclerosis (Review). Prescriber 2008;18:59-65.

Pauling JD, Gunawardena H, Coghlan JG, Easaw J, Suntharalingam J, McHugh NJ. Pulmonary artery hypertension as the presenting feature of systemic sclerosis sine scleroderma. Rheumatology 2008;47:1431-1432.

Selected Abstracts

Gunawardena H , Wedderburn LR ,Chinoy H, Betteridge ZE, North J, Cooper RG, Ramanan AV, Davidson JE, McHugh NJ, for the Juvenile Dermatomyositis Research Group UK. Novel autoantibodies targeting a p140 protein, a major autoantigen system in juvenile dermatomyositis; clinical and immunogenetic associations. Arthritis Rheum. 2008;58:S923.

Gunawardena H, Wedderburn LR ,Chinoy H, Betteridge ZE, North J, Cooper RG, Ramanan AV, Davidson JE, McHugh NJ, for the Juvenile Dermatomyositis Research Group UK. Novel autoantibodies targeting a p140 protein, a major autoantigen system in juvenile dermatomyositis. Paediatric Rheumatology 2008;6:S18.

Betteridge Z, Gunawardena H, Chinoy H, North J, Ollier W, Cooper R et al. Clinical and immunogenetic characteristics of patients with autoantibodies to small ubiquitin-like modifier activating enzyme in dermatomyositis. Arthritis Rheum 2008:58:S164.

Chinoy H, Platt H, Lamb JE, Betteridge Z, Gunawardena H, Fertig N, Davidson J, Oddis CV, McHugh NJ, Wedderburn LR, Ollier WE, Cooper RG. Genetic association study of NF-κB genes in UK caucasian adult and juvenile onset idiopathic inflammatory myopathy. Arthritis Rheum 2008;58:S162.

Gunawardena H, Betteridge Z, Owen P, McHugh NJ, Foley NM. Novel markers of connective tissue disease in patients with idiopathic interstitial pneumonia. European Respiratory Journal 2008;32:320s.

Gunawardena H, Betteridge Z and McHugh N. The prevalence and clinical associations of myositis specific and associated autoantibodies in idiopathic inflammatory myopathy. Rheumatology 2008;47:ii84.

Betteridge Z, Gunawardena H, Chinoy H, North J, Ollier W, Cooper R et al. Clinical and immunogenetic association of anti-SAE autoantibodies in patients with dermatomyositis. Rheumatology 2008;47:ii85.

Chinoy H, Platt H, Lamb J, Betteridge Z, Gunawardena H, Wedderburn LR et al. In UK caucasian patients with adult and juvenile onset myositis, the PTPN22 1858C→T allele represents a disease susceptibility gene which is independent of HLA-DRB1*03. Rheumatology 2008;47:ii13.

Betteridge ZE, Gunawardena H, North J, Slinn J, McHugh NJ Identification of a novel autoantibody in dermatomyositis directed against small ubiquitin-like modifier (SUMO-1) activating enzyme. Rheumatology 2007;46:i11.

Gunawardena H, North J, Wedderburn LR, Davidson JE, Betteridge ZE, Dunphy J, Chinoy H, Cooper RG, McHugh NJ. Clinical associations of anti-p155/140 autoantibodies in adult and juvenile dermatomyositis. Ann Rheum Dis 2007;66:S68.

Betteridge ZE, Gunawardena H, North J, Slinn J, McHugh NJ. Anti-Zo: A new autoantibody to phenylalanyl-transfer RNA synthetase associated with polymyositis and interstitial Pneumonia. Rheumatology 2007;46:i12.

North J, Gunawardena H, Wedderburn LR, Davidson JE, Betteridge ZE, Dunphy J, Chinoy H, Cooper RG, McHugh NJ. Autoantibodies to p155/140 in adult dermatomyositis are a major autoantigen target in juvenile dermatomyositis. Rheumatology 2007;46:i11.

Gunawardena H, Harris ND, Carmichael C, McHugh NJ. Microvascular responses in systemic sclerosis. Rheumatology 2007;46:i76.

Gunawardena H, Harris ND, Carmichael C, McHugh NJ. Endothelial responses using digital iontophoresis with laser doppler imaging in idiopathic inflammatory myopathy. Rheumatology 2007;46:i73.

Gunawardena H, Betteridge ZE, Robinson G, North J, Korendowych E, Foley NM, McHugh NJ. Serological associations and subtypes of diffuse parenchymal lung disease in scleroderma spectrum disorders and idiopathic inflammatory myopathy. Rheumatology 2007;46:i76.

Gunawardena H, Betteridge ZE , North J, Robinson G, Foley NM, McHugh NJ Screening for anti-synthetase autoantibodies in patients with interstitial pneumonia. Rheumatology 2007;46:i73.

Gunawardena H, Harris ND, Carmichael C, McHugh NJ. Vascular regulatory responses in secondary Raynaud's phenomenon. Arthritis Rheum 2006;54:S742.

Gunawardena H, Robinson G, Betteridge Z, North J, Carmichael C, Foley NM, McHugh NJ. Interstitial pneumonia in polymyositis and dermatomyositis. Arthritis Rheum 2006;54:S660.

Betteridge Z, North J, Gunawardena H, Slinn J, McHugh NJ. Identification of novel autoantibodies to SUMO-E1 in dermatomyositis. Arthritis Rheum 2006;54:S660.

Gunawardena H, Carmichael C, Foley NM, McHugh NJ. Interstitial lung disease in systemic sclerosis. Rheumatology 2006; 45:i152.

Gunawardena H, Chan M, Carmichael C, Mansfield R, Foley NM, McHugh NJ. Screening for isolated pulmonary hypertension in systemic sclerosis. Rheumatology 2006;45:i152.

Gunawardena H, Carmichael C, Foley NM, McHugh NJ. Lung involvement in Sjogren's syndrome. Rheumatology 2006;45:i156.

References

1. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N.Engl.J.Med. 1975;292(7):344-347.

2. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N.Engl.J.Med. 1975;292(8):403-407.

3. Cooper GS, Miller FW, Pandey JP. The role of genetic factors in autoimmune disease: implications for environmental research. Environ Health Perspect 1999;107 Suppl 5:693-700.

4. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv.Immunol. 1989;44:93-151.

5. Mimori T. Autoantibodies in connective tissue diseases: clinical significance and analysis of target autoantigens. Intern.Med. 1999;38(7):523-532.

6. Love LA, Leff RL, Fraser DD, Targoff IN, Dalakas M, Plotz PH, et al. A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine (Baltimore) 1991;70(6):360-374.

7. Targoff IN. Autoantibodies and their significance in myositis. Curr.Rheumatol.Rep. 2008;10(4):333-340.

8. O'Hanlon TP, Carrick DM, Targoff IN, Arnett FC, Reveille JD, Carrington M, et al. Immunogenetic risk and protective factors for the idiopathic inflammatory myopathies: distinct HLA-A, -B, -Cw, -DRB1, and -DQA1 allelic profiles distinguish European American patients with different myositis autoantibodies. Medicine (Baltimore) 2006;85(2):111-127.

9. Chinoy H, Salway F, Fertig N, Shephard N, Tait BD, Thomson W, et al. In adult onset myositis, the presence of interstitial lung disease and myositis specific/associated antibodies are governed by HLA class II haplotype, rather than by myositis subtype. Arthritis Res.Ther. 2006;8(1):R13.

10. Wedderburn LR, McHugh NJ, Chinoy H, Cooper RG, Salway F, Ollier WE, et al. HLA class II haplotype and autoantibody associations in children with juvenile dermatomyositis and juvenile dermatomyositis-scleroderma overlap. Rheumatology.(Oxford) 2007;46(12):1786-1791.

11. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum. 1980;23(5):581-590.

12. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25(11):1271-1277.

13. Tanimoto K, Nakano K, Kano S, Mori S, Ueki H, Nishitani H, et al. Classification criteria for polymyositis and dermatomyositis. J Rheumatol 1995;22(4):668-74.

14. Griggs RC, Askanas V, DiMauro S, Engel A, Karpati G, Mendell JR, et al. Inclusion body myositis and myopathies. Ann Neurol 1995;38(5):705-13.

15. Needham M, Mastaglia FL. Inclusion body myositis: current pathogenetic concepts and diagnostic and therapeutic approaches. Lancet Neurol 2007;6(7):620-31.

16. Mastaglia FL, Phillips BA. Idiopathic inflammatory myopathies: epidemiology, classification, and diagnostic criteria. Rheum Dis Clin North Am 2002;28(4):723-41.

17. Dalakas MC, Hohlfeld R. Polymyositis and dermatomyositis. Lancet 2003;362(9388):971-82.

18. Oddis CV, Conte CG, Steen VD, Medsger TA, Jr. Incidence of polymyositis-dermatomyositis: a 20-year study of hospital diagnosed cases in Allegheny County, PA 1963-1982. J Rheumatol 1990;17(10):1329-34.

19. Mendez EP, Lipton R, Ramsey-Goldman R, Roettcher P, Bowyer S, Dyer A, et al. US incidence of juvenile dermatomyositis, 1995-1998: results from the National Institute of Arthritis and Musculoskeletal and Skin Diseases Registry. Arthritis Rheum 2003;49(3):300-5.

20. Symmons DP, Sills JA, Davis SM. The incidence of juvenile dermatomyositis: results from a nation-wide study. Br J Rheumatol 1995;34(8):732-6.

21. Ramanan AV, Feldman BM. Clinical features and outcomes of juvenile dermatomyositis and other childhood onset myositis syndromes. Rheum.Dis.Clin.North Am. 2002;28(4):833-857.

22. Ramanan AV, Feldman BM. Clinical outcomes in juvenile dermatomyositis. Curr Opin Rheumatol 2002;14(6):658-62.

23. Huber A, Feldman BM. Long-term outcomes in juvenile dermatomyositis: how did we get here and where are we going? Curr Rheumatol Rep 2005;7(6):441-6.

24. McCann LJ, Juggins AD, Maillard SM, Wedderburn LR, Davidson JE, Murray KJ, et al. The Juvenile Dermatomyositis National Registry and Repository (UK and

Ireland)--clinical characteristics of children recruited within the first 5 yr. Rheumatology.(Oxford) 2006;45(10):1255-1260.

25. Pilkington CA, Wedderburn LR. Paediatric idiopathic inflammatory muscle disease: recognition and management. Drugs 2005;65(10):1355-65.

26. Euwer RL, Sontheimer RD. Dermatologic aspects of myositis. Curr Opin Rheumatol 1994;6(6):583-9.

27. Danko K, Ponyi A, Constantin T, Borgulya G, Szegedi G. Long-term survival of patients with idiopathic inflammatory myopathies according to clinical features: a longitudinal study of 162 cases. Medicine (Baltimore) 2004;83(1):35-42.

28. Fathi M, Dastmalchi M, Rasmussen E, Lundberg IE, Tornling G. Interstitial lung disease, a common manifestation of newly diagnosed polymyositis and dermatomyositis. Ann.Rheum.Dis. 2004;63(3):297-301.

29. Fathi M, Lundberg IE. Interstitial lung disease in polymyositis and dermatomyositis. Curr Opin Rheumatol 2005;17(6):701-6.

30. Fathi M, Lundberg IE, Tornling G. Pulmonary complications of polymyositis and dermatomyositis. Semin Respir Crit Care Med 2007;28(4):451-8.

31. Kang EH, Lee EB, Shin KC, Im CH, Chung DH, Han SK, et al. Interstitial lung disease in patients with polymyositis, dermatomyositis and amyopathic dermatomyositis. Rheumatology.(Oxford) 2005;44(10):1282-1286.

32. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med 2000;161(2 Pt 1):646-64.

33. Tansey D, Wells AU, Colby TV, Ip S, Nikolakoupolou A, du Bois RM, et al. Variations in histological patterns of interstitial pneumonia between connective tissue disorders and their relationship to prognosis. Histopathology 2004;44(6):585-96.

34. Bjoraker JA, Ryu JH, Edwin MK, Myers JL, Tazelaar HD, Schroeder DR, et al. Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 1998;157(1):199-203.

35. Katzenstein AL, Fiorelli RF. Nonspecific interstitial pneumonia/fibrosis. Histologic features and clinical significance. Am J Surg Pathol 1994;18(2):136-47.

36. Kim EA, Lee KS, Johkoh T, Kim TS, Suh GY, Kwon OJ, et al. Interstitial lung diseases associated with collagen vascular diseases: radiologic and histopathologic findings. Radiographics 2002;22 Spec No:S151-65.

37. MacDonald SL, Rubens MB, Hansell DM, Copley SJ, Desai SR, du Bois RM, et al. Nonspecific interstitial pneumonia and usual interstitial pneumonia: comparative appearances at and diagnostic accuracy of thin-section CT. Radiology 2001;221(3):600-5.

38. Douglas WW, Tazelaar HD, Hartman TE, Hartman RP, Decker PA, Schroeder DR, et al. Polymyositis-dermatomyositis-associated interstitial lung disease. Am J Respir Crit Care Med 2001;164(7):1182-5.

39. Fathi M, Vikgren J, Boijsen M, Tylen U, Jorfeldt L, Tornling G, et al. Interstitial lung disease in polymyositis and dermatomyositis: longitudinal evaluation by pulmonary function and radiology. Arthritis Rheum 2008;59(5):677-85.

40. Gerami P, Schope JM, McDonald L, Walling HW, Sontheimer RD. A systematic review of adult-onset clinically amyopathic dermatomyositis

(dermatomyositis sine myositis): a missing link within the spectrum of the idiopathic inflammatory myopathies. J.Am.Acad.Dermatol. 2006;54(4):597-613.

41. Ye S, Chen XX, Lu XY, Wu MF, Deng Y, Huang WQ, et al. Adult clinically amyopathic dermatomyositis with rapid progressive interstitial lung disease: a retrospective cohort study. Clin.Rheumatol. 2007;26(10):1647-1654.

42. Friedman AW, Targoff IN, Arnett FC. Interstitial lung disease with autoantibodies against aminoacyl-tRNA synthetases in the absence of clinically apparent myositis. Semin.Arthritis Rheum. 1996;26(1):459-467.

43. Mamyrova G, Kleiner DE, James-Newton L, Shaham B, Miller FW, Rider LG. Late-onset gastrointestinal pain in juvenile dermatomyositis as a manifestation of ischemic ulceration from chronic endarteropathy. Arthritis Rheum. 2007;57(5):881-884.

44. Lundberg IE. The heart in dermatomyositis and polymyositis. Rheumatology (Oxford) 2006;45 Suppl 4:iv18-21.

45. Lundberg IE. Cardiac involvement in autoimmune myositis and mixed connective tissue disease. Lupus 2005;14(9):708-12.

46. Targoff IN. Myositis specific autoantibodies. Curr Rheumatol Rep 2006;8(3):196-203.

47. Marie I, Hachulla E, Cherin P, Dominique S, Hatron PY, Hellot MF, et al. Interstitial lung disease in polymyositis and dermatomyositis. Arthritis Rheum. 2002;47(6):614-622.

48. Yoshifuji H, Fujii T, Kobayashi S, Imura Y, Fujita Y, Kawabata D, et al. Anti-aminoacyl-tRNA synthetase antibodies in clinical course prediction of interstitial lung

disease complicated with idiopathic inflammatory myopathies. Autoimmunity 2006;39(3):233-241.

49. Sigurgeirsson B, Lindelof B, Edhag O, Allander E. Risk of cancer in patients with dermatomyositis or polymyositis. A population-based study. N Engl J Med 1992;326(6):363-7.

50. Callen JP. When and how should the patient with dermatomyositis or amyopathic dermatomyositis be assessed for possible cancer? Arch Dermatol 2002;138(7):969-71.

51. Callen JP. Relation between dermatomyositis and polymyositis and cancer. Lancet 2001;357(9250):85-6.

52. Callen JP. Relationship of cancer to inflammatory muscle diseases. Dermatomyositis, polymyositis, and inclusion body myositis. Rheum Dis Clin North Am 1994;20(4):943-53.

53. Hill CL, Zhang Y, Sigurgeirsson B, Pukkala E, Mellemkjaer L, Airio A, et al. Frequency of specific cancer types in dermatomyositis and polymyositis: a population-based study. Lancet 2001;357(9250):96-100.

54. Levine SM. Cancer and myositis: new insights into an old association. Curr Opin Rheumatol 2006;18(6):620-4.

55. Buchbinder R, Forbes A, Hall S, Dennett X, Giles G. Incidence of malignant disease in biopsy-proven inflammatory myopathy. A population-based cohort study. Ann Intern Med 2001;134(12):1087-95.

56. Buchbinder R, Hill CL. Malignancy in patients with inflammatory myopathy. Curr Rheumatol Rep 2002;4(5):415-26.

57. Rider LG, Miller FW. Classification and treatment of the juvenile idiopathic inflammatory myopathies. Rheum Dis Clin North Am 1997;23(3):619-55.

58. Bunch TW. Prednisone and azathioprine for polymyositis: long-term followup. Arthritis Rheum 1981;24(1):45-8.

59. Bunch TW, Worthington JW, Combs JJ, Ilstrup DM, Engel AG. Azathioprine with prednisone for polymyositis. A controlled, clinical trial. Ann Intern Med 1980;92(3):365-9.

60. Villalba L, Hicks JE, Adams EM, Sherman JB, Gourley MF, Leff RL, et al. Treatment of refractory myositis: a randomized crossover study of two new cytotoxic regimens. Arthritis Rheum 1998;41(3):392-9.

61. Choy EH, Hoogendijk JE, Lecky B, Winer JB. Immunosuppressant and immunomodulatory treatment for dermatomyositis and polymyositis. Cochrane Database Syst Rev 2005(3):CD003643.

62. Vencovsky J, Jarosova K, Machacek S, Studynkova J, Kafkova J, Bartunkova J, et al. Cyclosporine A versus methotrexate in the treatment of polymyositis and dermatomyositis. Scand J Rheumatol 2000;29(2):95-102.

63. Dalakas MC, Illa I, Dambrosia JM, Soueidan SA, Stein DP, Otero C, et al. A controlled trial of high-dose intravenous immune globulin infusions as treatment for dermatomyositis. N Engl J Med 1993;329(27):1993-2000.

64. Levine TD. Rituximab in the treatment of dermatomyositis: an open-label pilot study. Arthritis Rheum. 2005;52(2):601-607.

65. Edge JC, Outland JD, Dempsey JR, Callen JP. Mycophenolate mofetil as an effective corticosteroid-sparing therapy for recalcitrant dermatomyositis. Arch Dermatol 2006;142(1):65-9.

66. Majithia V, Harisdangkul V. Mycophenolate mofetil (CellCept): an alternative therapy for autoimmune inflammatory myopathy. Rheumatology (Oxford) 2005;44(3):386-9.

67. Pisoni CN, Cuadrado MJ, Khamashta MA, Hughes GR, D'Cruz DP. Mycophenolate mofetil treatment in resistant myositis. Rheumatology (Oxford) 2007;46(3):516-8.

68. Oddis CV, Sciurba FC, Elmagd KA, Starzl TE. Tacrolimus in refractory polymyositis with interstitial lung disease. Lancet 1999;353(9166):1762-3.

69. Wilkes MR, Sereika SM, Fertig N, Lucas MR, Oddis CV. Treatment of antisynthetase-associated interstitial lung disease with tacrolimus. Arthritis Rheum 2005;52(8):2439-46.

70. Agarwal S, Kiely PD. Two simple, reliable and valid tests of proximal muscle function, and their application to the management of idiopathic inflammatory myositis. Rheumatology (Oxford) 2006;45(7):874-9.

71. Miller FW, Rider LG, Chung YL, Cooper R, Danko K, Farewell V, et al. Proposed preliminary core set measures for disease outcome assessment in adult and juvenile idiopathic inflammatory myopathies. Rheumatology (Oxford) 2001;40(11):1262-73.

72. Sultan SM, Allen E, Oddis CV, Kiely P, Cooper RG, Lundberg IE, et al. Reliability and validity of the myositis disease activity assessment tool. Arthritis Rheum 2008;58(11):3593-9.

73. Emslie-Smith AM, Engel AG. Microvascular changes in early and advanced dermatomyositis: a quantitative study. Ann Neurol 1990;27(4):343-56.

74. Crowson AN, Magro CM. The role of microvascular injury in the pathogenesis of cutaneous lesions of dermatomyositis. Hum Pathol 1996;27(1):15-9.

75. Dalakas MC, Sivakumar K. The immunopathologic and inflammatory differences between dermatomyositis, polymyositis and sporadic inclusion body myositis. Curr Opin Neurol 1996;9(3):235-9.

76. Engel AG, Arahata K. Monoclonal antibody analysis of mononuclear cells in myopathies. II: Phenotypes of autoinvasive cells in polymyositis and inclusion body myositis. Ann Neurol 1984;16(2):209-15.

77. Goebels N, Michaelis D, Engelhardt M, Huber S, Bender A, Pongratz D, et al. Differential expression of perforin in muscle-infiltrating T cells in polymyositis and dermatomyositis. J Clin Invest 1996;97(12):2905-10.

78. Greenberg SA. A gene expression approach to study perturbed pathways in myositis. Curr Opin Rheumatol 2007;19(6):536-41.

79. Wenzel J, Schmidt R, Proelss J, Zahn S, Bieber T, Tuting T. Type I interferon-associated skin recruitment of CXCR3+ lymphocytes in dermatomyositis. Clin Exp Dermatol 2006;31(4):576-82.

80. Eloranta ML, Barbasso HS, Ulfgren AK, Ronnblom L, Alm GV, Lundberg IE. A possible mechanism for endogenous activation of the type I interferon system in myositis patients with anti-Jo-1 or anti-Ro 52/anti-Ro 60 autoantibodies. Arthritis Rheum. 2007;56(9):3112-3124.

81. Greenberg SA, Bradshaw EM, Pinkus JL, Pinkus GS, Burleson T, Due B, et al. Plasma cells in muscle in inclusion body myositis and polymyositis. Neurology 2005;65(11):1782-7.

82. Bradshaw EM, Orihuela A, McArdel SL, Salajegheh M, Amato AA, Hafler DA, et al. A local antigen-driven humoral response is present in the inflammatory myopathies. J Immunol 2007;178(1):547-56.

83. Nagaraju K, Raben N, Loeffler L, Parker T, Rochon PJ, Lee E, et al. Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. Proc Natl Acad Sci U S A 2000;97(16):9209-14.

84. Tajima Y, Moriwaka F, Tashiro K. Temporal alterations of immunohistochemical findings in polymyositis. Intern Med 1994;33(5):263-70.

85. Dalakas MC. Mechanisms of disease: signaling pathways and immunobiology of inflammatory myopathies. Nat Clin Pract Rheumatol 2006;2(4):219-27.

86. Nagaraju K, Casciola-Rosen L, Lundberg I, Rawat R, Cutting S, Thapliyal R, et al. Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. Arthritis Rheum. 2005;52(6):1824-1835.

87. Shamim EA, Rider LG, Miller FW. Update on the genetics of the idiopathic inflammatory myopathies. Curr Opin Rheumatol 2000;12(6):482-91.

88. Chinoy H, Ollier WE, Cooper RG. Have recent immunogenetic investigations increased our understanding of disease mechanisms in the idiopathic inflammatory myopathies? 11. Curr.Opin.Rheumatol. 2004;16(6):707-713.

89. O'Hanlon TP, Carrick DM, Arnett FC, Reveille JD, Carrington M, Gao X, et al. Immunogenetic risk and protective factors for the idiopathic inflammatory myopathies: distinct HLA-A, -B, -Cw, -DRB1 and -DQA1 allelic profiles and motifs

define clinicopathologic groups in caucasians. Medicine (Baltimore) 2005;84(6):338-349.

90. O'Hanlon TP, Rider LG, Mamyrova G, Targoff IN, Arnett FC, Reveille JD, et al. HLA polymorphisms in African Americans with idiopathic inflammatory myopathy: allelic profiles distinguish patients with different clinical phenotypes and myositis autoantibodies. Arthritis Rheum. 2006;54(11):3670-3681.

91. Shamim EA, Rider LG, Pandey JP, O'Hanlon TP, Jara LJ, Samayoa EA, et al. Differences in idiopathic inflammatory myopathy phenotypes and genotypes between Mesoamerican Mestizos and North American Caucasians: ethnogeographic influences in the genetics and clinical expression of myositis. Arthritis Rheum. 2002;46(7):1885-1893.

92. Chinoy H, Salway F, John S, Fertig N, Tait BD, Oddis CV, et al. Tumour necrosis factor-alpha single nucleotide polymorphisms are not independent of HLA class I in UK Caucasians with adult onset idiopathic inflammatory myopathies. Rheumatology.(Oxford) 2007;46(9):1411-1416.

93. Leff RL, Burgess SH, Miller FW, Love LA, Targoff IN, Dalakas MC, et al. Distinct seasonal patterns in the onset of adult idiopathic inflammatory myopathy in patients with anti-Jo-1 and anti-signal recognition particle autoantibodies. Arthritis Rheum. 1991;34(11):1391-1396.

94. Sarkar K, Weinberg CR, Oddis CV, Medsger TA, Jr., Plotz PH, Reveille JD, et al. Seasonal influence on the onset of idiopathic inflammatory myopathies in serologically defined groups. Arthritis Rheum. 2005;52(8):2433-2438.

95. Vegosen LJ, Weinberg CR, O'Hanlon TP, Targoff IN, Miller FW, Rider LG. Seasonal birth patterns in myositis subgroups suggest an etiologic role of early environmental exposures. Arthritis Rheum. 2007;56(8):2719-2728.

96. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J.Exp.Med. 1994;179(4):1317-1330.

97. Casciola-Rosen L, Rosen A. Ultraviolet light-induced keratinocyte apoptosis: a potential mechanism for the induction of skin lesions and autoantibody production in LE. Lupus 1997;6(2):175-80.

98. Andrade F, Casciola-Rosen LA, Rosen A. Generation of novel covalent RNA-protein complexes in cells by ultraviolet B irradiation: implications for autoimmunity. Arthritis Rheum 2005;52(4):1160-70.

99. Hengstman GJ, van Venrooij WJ, Vencovsky J, Moutsopoulos HM, van Engelen BG. The relative prevalence of dermatomyositis and polymyositis in Europe exhibits a latitudinal gradient. Ann Rheum Dis 2000;59(2):141-2.

100. Tan EM. Antinuclear antibodies: diagnostic markers and clues to the basis of systemic autoimmunity. Pediatr Infect Dis J 1988;7(5 Suppl):S3-9.

101. Tan EM. Antinuclear antibodies in diagnosis and management. Hosp Pract (Off Ed) 1983;18(1):79-84.

102. Andrade F, Casciola-Rosen L, Rosen A. Apoptosis in systemic lupus erythematosus. Clinical implications. Rheum Dis Clin North Am 2000;26(2):215-27, v.

103. Andrade F, Casciola-Rosen LA, Rosen A. Granzyme B-induced cell death. Acta Haematol 2004;111(1-2):28-41.

104. Casciola-Rosen L, Andrade F, Ulanet D, Wong WB, Rosen A. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J.Exp.Med. 1999;190(6):815-826.

105. Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. J.Exp.Med. 1997;185(1):71-79.

106. Duan-Porter WD, Casciola-Rosen L, Rosen A. Autoantigens: the critical partner in initiating and propagating systemic autoimmunity. Ann.N.Y.Acad.Sci. 2005;1062:127-136.

107. Casciola-Rosen L, Nagaraju K, Plotz P, Wang K, Levine S, Gabrielson E, et al. Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy. J.Exp.Med. 2005;201(4):591-601.

108. Levine SM, Raben N, Xie D, Askin FB, Tuder R, Mullins M, et al. Novel conformation of histidyl-transfer RNA synthetase in the lung: the target tissue in Jo-1 autoantibody-associated myositis. Arthritis Rheum. 2007;56(8):2729-2739.

109. Targoff IN. Laboratory testing in the diagnosis and management of idiopathic inflammatory myopathies. Rheum Dis Clin North Am 2002;28(4):859-90, viii.

110. Targoff IN. Idiopathic inflammatory myopathy: autoantibody update. Curr Rheumatol Rep 2002;4(5):434-41.

111. Nishikai M, Reichlin M. Heterogeneity of precipitating antibodies in polymyositis and dermatomyositis. Characterization of the Jo-1 antibody system. Arthritis Rheum. 1980;23(8):881-888.

112. Mimori T, Imura Y, Nakashima R, Yoshifuji H. Autoantibodies in idiopathic inflammatory myopathy: an update on clinical and pathophysiological significance. Curr.Opin.Rheumatol. 2007;19(6):523-529.

113. Brouwer R, Hengstman GJ, Vree EW, Ehrfeld H, Bozic B, Ghirardello A, et al. Autoantibody profiles in the sera of European patients with myositis. Ann.Rheum.Dis. 2001;60(2):116-123.

114. Mathews MB, Reichlin M, Hughes GR, Bernstein RM. Anti-threonyl-tRNA synthetase, a second myositis-related autoantibody. J.Exp.Med. 1984;160(2):420-434.

115. Bunn CC, Bernstein RM, Mathews MB. Autoantibodies against alanyl-tRNA synthetase and tRNAAla coexist and are associated with myositis. J.Exp.Med. 1986;163(5):1281-1291.

116. Targoff IN, Trieu EP, Plotz PH, Miller FW. Antibodies to glycyl-transfer RNA synthetase in patients with myositis and interstitial lung disease. Arthritis Rheum. 1992;35(7):821-830.

117. Targoff IN, Trieu EP, Miller FW. Reaction of anti-OJ autoantibodies with components of the multi-enzyme complex of aminoacyl-tRNA synthetases in addition to isoleucyl-tRNA synthetase. J.Clin.Invest 1993;91(6):2556-2564.

118. Hirakata M, Suwa A, Nagai S, Kron MA, Trieu EP, Mimori T, et al. Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J.Immunol. 1999;162(4):2315-2320.

119. Hashish L, Trieu EP, Sadanandan P, Targoff IN. Identification of autoantibodies to tyrosyl-tRNA synthetase in dermatomyositis with features consistent with antisynthetase syndrome (abstract). Arthritis Rheum 2005;52:S312.

120. Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Anti-synthetase syndrome: a new autoantibody to phenylalanyl transfer RNA synthetase (anti-Zo)

associated with polymyositis and interstitial pneumonia. Rheumatology.(Oxford) 2007;46(6):1005-1008.

121. Reeves WH, Nigam SK, Blobel G. Human autoantibodies reactive with the signal-recognition particle. Proc.Natl.Acad.Sci.U.S.A 1986;83(24):9507-9511.

122. Targoff IN, Johnson AE, Miller FW. Antibody to signal recognition particle in polymyositis. Arthritis Rheum. 1990;33(9):1361-1370.

123. Hengstman GJ, ter Laak HJ, Vree Egberts WT, Lundberg IE, Moutsopoulos HM, Vencovsky J, et al. Anti-signal recognition particle autoantibodies: marker of a necrotising myopathy. Ann.Rheum.Dis. 2006;65(12):1635-1638.

124. Miller T, Al-Lozi MT, Lopate G, Pestronk A. Myopathy with antibodies to the signal recognition particle: clinical and pathological features. J.Neurol.Neurosurg.Psychiatry 2002;73(4):420-428.

125. Rider LG, Miller FW, Targoff IN, Sherry DD, Samayoa E, Lindahl M, et al. A broadened spectrum of juvenile myositis. Myositis-specific autoantibodies in children. Arthritis Rheum. 1994;37(10):1534-1538.

126. Targoff IN, Reichlin M. The association between Mi-2 antibodies and dermatomyositis. Arthritis Rheum. 1985;28(7):796-803.

127. Feldman BM, Reichlin M, Laxer RM, Targoff IN, Stein LD, Silverman ED. Clinical significance of specific autoantibodies in juvenile dermatomyositis. J.Rheumatol. 1996;23(10):1794-1797.

128. Ghirardello A, Zampieri S, Iaccarino L, Tarricone E, Bendo R, Gambari PF, et al. Anti-Mi-2 antibodies. Autoimmunity 2005;38(1):79-83.

129. Wang HB, Zhang Y. Mi2, an auto-antigen for dermatomyositis, is an ATP-dependent nucleosome remodeling factor. Nucleic Acids Res. 2001;29(12):2517-2521.

130. Sato S, Hirakata M, Kuwana M, Suwa A, Inada S, Mimori T, et al. Autoantibodies to a 140-kd polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. Arthritis Rheum. 2005;52(5):1571-1576.

131. Sato S, Hoshino K, Satoh T, Fujita T, Kawakami Y, Kuwana M. RNA helicase encoded by melanoma differentiation-associated gene 5 is a major autoantigen in patients with clinically amyopathic dermatomyositis: Association with rapidly progressive interstitial lung disease. Arthritis Rheum 2009;60(7):2193-200.

132. Takeuchi O, Akira S. MDA5/RIG-I and virus recognition. Curr.Opin.Immunol. 2008;20(1):17-22.

133. Katsumata Y, Ridgway WM, Oriss T, Gu X, Chin D, Wu Y, et al. Species-specific immune responses generated by histidyl-tRNA synthetase immunization are associated with muscle and lung inflammation. J.Autoimmun. 2007;29(2-3):174-186.

134. Kashiwagi M, Morgan BA, Georgopoulos K. The chromatin remodeler Mi-2beta is required for establishment of the basal epidermis and normal differentiation of its progeny. Development 2007;134(8):1571-1582.

135. Howard OM, Dong HF, Yang D, Raben N, Nagaraju K, Rosen A, et al. Histidyl-tRNA synthetase and asparaginyl-tRNA synthetase, autoantigens in myositis, activate chemokine receptors on T lymphocytes and immature dendritic cells. J.Exp.Med. 2002;196(6):781-791.

136. Mozaffar T, Pestronk A. Myopathy with anti-Jo-1 antibodies: pathology in perimysium and neighbouring muscle fibres. J.Neurol.Neurosurg.Psychiatry 2000;68(4):472-478.

137. Casciola-Rosen L. Autoimmune myositis: new concepts for disease initiation and propagation. Curr Opin Rheumatol 2005;17(6):699-700.

138. Suber TL, Casciola-Rosen L, Rosen A. Mechanisms of disease: autoantigens as clues to the pathogenesis of myositis. Nat.Clin.Pract.Rheumatol. 2008;4(4):201-209.

139. Troyanov Y, Targoff IN, Tremblay JL, Goulet JR, Raymond Y, Senecal JL. Novel classification of idiopathic inflammatory myopathies based on overlap syndrome features and autoantibodies: analysis of 100 French Canadian patients. Medicine (Baltimore) 2005;84(4):231-249.

140. Targoff IN, Miller FW, Medsger TA, Jr., Oddis CV. Classification criteria for the idiopathic inflammatory myopathies. Curr Opin Rheumatol 1997;9(6):527-35.

141. Vitali C, Bombardieri S, Moutsopoulos HM, Balestrieri G, Bencivelli W, Bernstein RM, et al. Preliminary criteria for the classification of Sjogren's syndrome. Results of a prospective concerted action supported by the European Community. Arthritis Rheum 1993;36(3):340-7.

142. Alarcon-Segovia D. Mixed connective tissue disease and overlap syndromes. Clin Dermatol 1994;12(2):309-16.

143. Targoff IN, Mamyrova G, Trieu EP, Perurena O, Koneru B, O'Hanlon TP, et al. A novel autoantibody to a 155-kd protein is associated with dermatomyositis. Arthritis Rheum. 2006;54(11):3682-3689.

144. Targoff IN, Arnett FC. Clinical manifestations in patients with antibody to PL-12 antigen (alanyl-tRNA synthetase). Am.J.Med. 1990;88(3):241-251.

145. Yamasaki Y, Yamada H, Nozaki T, Akaogi J, Nichols C, Lyons R, et al. Unusually high frequency of autoantibodies to PL-7 associated with milder muscle disease in Japanese patients with polymyositis/dermatomyositis. Arthritis Rheum. 2006;54(6):2004-2009.

146. Sato S, Kuwana M, Hirakata M. Clinical characteristics of Japanese patients with anti-OJ (anti-isoleucyl-tRNA synthetase) autoantibodies. Rheumatology.(Oxford) 2007;46(5):842-845.

147. Hirakata M, Suwa A, Takada T, Sato S, Nagai S, Genth E, et al. Clinical and immunogenetic features of patients with autoantibodies to asparaginyl-transfer RNA synthetase. Arthritis Rheum. 2007;56(4):1295-1303.

148. Yang Y, Fujita J, Tokuda M, Bandoh S, Ishida T. Chronological evaluation of the onset of histologically confirmed interstitial pneumonia associated with polymyositis/dermatomyositis. Intern.Med. 2002;41(12):1135-1141.

149. Ghirardello A, Zampieri S, Tarricone E, Iaccarino L, Bendo R, Briani C, et al. Clinical implications of autoantibody screening in patients with autoimmune myositis. Autoimmunity 2006;39(3):217-221.

150. Kalluri M, Sahn SA, Oddis CV, Gharib SL, Christopher-Stine L, Danoff SK, et al. Clinical Profile of Anti-PL-12 Autoantibody: Cohort Study and Review of the Literature. Chest 2009.

151. Tillie-Leblond I, Wislez M, Valeyre D, Crestani B, Rabbat A, Israel-Biet D, et al. Interstitial lung disease and anti-Jo-1 antibodies: difference between acute and gradual onset. Thorax 2008;63(1):53-59.

152. Matsushita T, Hasegawa M, Fujimoto M, Hamaguchi Y, Komura K, Hirano T, et al. Clinical evaluation of anti-aminoacyl tRNA synthetase antibodies in Japanese patients with dermatomyositis. J.Rheumatol. 2007;34(5):1012-1018.

153. Suda T, Fujisawa T, Enomoto N, Nakamura Y, Inui N, Naito T, et al. Interstitial lung diseases associated with amyopathic dermatomyositis. Eur.Respir.J. 2006;28(5):1005-1012.

154. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441(7089):101-5.

155. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, et al. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J.Virol. 2008;82(1):335-345.

156. Nakashima R, Imura Y, Kobayashi S, Yukawa N, Yoshifuji H, Nojima T, et al. The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. Rheumatology (Oxford) 2009.

157. Kaji K, Fujimoto M, Hasegawa M, Kondo M, Saito Y, Komura K, et al. Identification of a novel autoantibody reactive with 155 and 140 kDa nuclear proteins in patients with dermatomyositis: an association with malignancy. Rheumatology.(Oxford) 2007;46(1):25-28.

158. Chinoy H, Fertig N, Oddis CV, Ollier WE, Cooper RG. The diagnostic utility of myositis autoantibody testing for predicting the risk of cancer-associated myositis. Ann.Rheum.Dis. 2007;66(10):1345-1349.

159. Targoff IN, Trieu EP, Levy-Neto M, Prasertsuntarsai T, Miller FW. Autoantibodies to transcriptional intermediary factor 1-gamma (TIF1-g) in dermatomyositis (abstract). Arthritis Rheum 2006;54:S518.

160. Betteridge Z, Gunawardena H, North J, Slinn J, McHugh N. Identification of a novel autoantibody directed against small ubiquitin-like modifier activating enzyme in dermatomyositis. Arthritis Rheum. 2007;56(9):3132-3137.

161. Gunawardena H, North J, Wedderburn L, Davidson J, Betteridge ZE, Dunphy J, et al. Clinical associations of anti-p155/140 autoantibodies in adult and juvenile dermatomyositis (abstract). Ann Rheum Dis 2007;66:S68.

162. Betteridge ZE, Gunawardena H, Chinoy H, North J, Ollier WE, Cooper RG, et al. Clinical and HLA-class II haplotype associations of autoantibodies to small ubiquitin-like modifier enzyme, a dermatomyositis-specific autoantigen target, in UK adult-onset Caucasian myositis. Ann.Rheum.Dis. 2008.

163. Cerini C, Kerjan P, Astier M, Gratecos D, Mirande M, Semeriva M. A component of the multisynthetase complex is a multifunctional aminoacyl-tRNA synthetase. EMBO J 1991;10(13):4267-77.

164. Andrade F, Bull HG, Thornberry NA, Ketner GW, Casciola-Rosen LA, Rosen A. Adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death. Immunity 2001;14(6):751-61.

165. Doyle HA, Mamula MJ. Post-translational protein modifications in antigen recognition and autoimmunity. Trends Immunol 2001;22(8):443-9.

166. Gill G. SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? Genes Dev 2004;18(17):2046-59.

167. Pichler A, Melchior F. Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. Traffic 2002;3(6):381-7.

168. Dohmen RJ. SUMO protein modification. Biochim.Biophys.Acta 2004;1695(1-3):113-131.

169. Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, Kolli N, et al. Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. Mol Cell Biol 2004;24(1):84-95.

170. Franz JK, Pap T, Hummel KM, Nawrath M, Aicher WK, Shigeyama Y, et al. Expression of sentrin, a novel antiapoptotic molecule, at sites of synovial invasion in rheumatoid arthritis. Arthritis Rheum 2000;43(3):599-607.

171. Janka C, Selmi C, Gershwin ME, Will H, Sternsdorf T. Small ubiquitin-related modifiers: A novel and independent class of autoantigens in primary biliary cirrhosis. Hepatology 2005;41(3):609-16.

172. Boki KA, Dourakis SP. Polymyositis associated with primary biliary cirrhosis. Clin Rheumatol 1995;14(3):375-8.

173. Milosevic M, Adams P. Primary biliary cirrhosis and polymyositis. J Clin Gastroenterol 1990;12(3):332-5.

174. Desterro JM, Rodriguez MS, Kemp GD, Hay RT. Identification of the enzyme required for activation of the small ubiquitin-like protein SUMO-1. J Biol Chem 1999;274(15):10618-24.

175. Lois LM, Lima CD. Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. EMBO J 2005;24(3):439-51.

176. Azuma Y, Tan SH, Cavenagh MM, Ainsztein AM, Saitoh H, Dasso M. Expression and regulation of the mammalian SUMO-1 E1 enzyme. FASEB J 2001;15(10):1825-7.

177. Pichler A, Gast A, Seeler JS, Dejean A, Melchior F. The nucleoporin RanBP2 has SUMO1 E3 ligase activity. Cell 2002;108(1):109-20.

178. Gong Z, Brackertz M, Renkawitz R. SUMO modification enhances p66-mediated transcriptional repression of the Mi-2/NuRD complex. Mol.Cell Biol. 2006;26(12):4519-4528.

179. Chun TH, Itoh H, Subramanian L, Iniguez-Lluhi JA, Nakao K. Modification of GATA-2 transcriptional activity in endothelial cells by the SUMO E3 ligase PIASy. Circ Res 2003;92(11):1201-8.

180. Kissel JT, Mendell JR, Rammohan KW. Microvascular deposition of complement membrane attack complex in dermatomyositis. N Engl J Med 1986;314(6):329-34.

181. Yan KP, Dolle P, Mark M, Lerouge T, Wendling O, Chambon P, et al. Molecular cloning, genomic structure, and expression analysis of the mouse transcriptional intermediary factor 1 gamma gene. Gene 2004;334:3-13.

182. Pachman LM, Lipton R, Ramsey-Goldman R, Shamiyeh E, Abbott K, Mendez EP, et al. History of infection before the onset of juvenile dermatomyositis: results from the National Institute of Arthritis and Musculoskeletal and Skin Diseases Research Registry. Arthritis Rheum 2005;53(2):166-72.

183. Pachman LM, Hayford JR, Chung A, Daugherty CA, Pallansch MA, Fink CW, et al. Juvenile dermatomyositis at diagnosis: clinical characteristics of 79 children. J Rheumatol 1998;25(6):1198-204.

184. Pachman LM, Abbott K, Sinacore JM, Amoruso L, Dyer A, Lipton R, et al. Duration of illness is an important variable for untreated children with juvenile dermatomyositis. J Pediatr 2006;148(2):247-53.

185. Pachman LM, Veis A, Stock S, Abbott K, Vicari F, Patel P, et al. Composition of calcifications in children with juvenile dermatomyositis: association with chronic cutaneous inflammation. Arthritis Rheum 2006;54(10):3345-50.

186. Tse S, Lubelsky S, Gordon M, Al Mayouf SM, Babyn PS, Laxer RM, et al. The arthritis of inflammatory childhood myositis syndromes. J Rheumatol 2001;28(1):192-7.

187. Huber AM, Lang B, LeBlanc CM, Birdi N, Bolaria RK, Malleson P, et al. Medium- and long-term functional outcomes in a multicenter cohort of children with juvenile dermatomyositis. Arthritis Rheum. 2000;43(3):541-549.

188. Huber AM, Feldman BM, Rennebohm RM, Hicks JE, Lindsley CB, Perez MD, et al. Validation and clinical significance of the Childhood Myositis Assessment Scale for assessment of muscle function in the juvenile idiopathic inflammatory myopathies. Arthritis Rheum 2004;50(5):1595-603.

189. Lovell DJ, Lindsley CB, Rennebohm RM, Ballinger SH, Bowyer SL, Giannini EH, et al. Development of validated disease activity and damage indices for the juvenile idiopathic inflammatory myopathies. II. The Childhood Myositis Assessment Scale (CMAS): a quantitative tool for the evaluation of muscle function. The Juvenile Dermatomyositis Disease Activity Collaborative Study Group. Arthritis Rheum 1999;42(10):2213-9.

190. Feldman BM, Ayling-Campos A, Luy L, Stevens D, Silverman ED, Laxer RM. Measuring disability in juvenile dermatomyositis: validity of the childhood health assessment questionnaire. J Rheumatol 1995;22(2):326-31.

191. Huber AM, Hicks JE, Lachenbruch PA, Perez MD, Zemel LS, Rennebohm RM, et al. Validation of the Childhood Health Assessment Questionnaire in the juvenile idiopathic myopathies. Juvenile Dermatomyositis Disease Activity Collaborative Study Group. J Rheumatol 2001;28(5):1106-11.

192. Wedderburn LR, Li CK. Paediatric idiopathic inflammatory muscle disease. Best Pract Res Clin Rheumatol 2004;18(3):345-58.

193. Huber AM, Dugan EM, Lachenbruch PA, Feldman BM, Perez MD, Zemel LS, et al. Preliminary validation and clinical meaning of the Cutaneous Assessment Tool in juvenile dermatomyositis. Arthritis Rheum 2008;59(2):214-21.

194. Huber AM, Dugan EM, Lachenbruch PA, Feldman BM, Perez MD, Zemel LS, et al. The Cutaneous Assessment Tool: development and reliability in juvenile idiopathic inflammatory myopathy. Rheumatology (Oxford) 2007;46(10):1606-11.

195. Maillard SM, Jones R, Owens C, Pilkington C, Woo P, Wedderburn LR, et al. Quantitative assessment of MRI T2 relaxation time of thigh muscles in juvenile dermatomyositis. Rheumatology (Oxford) 2004;43(5):603-8.

196. Kimball AB, Summers RM, Turner M, Dugan EM, Hicks J, Miller FW, et al. Magnetic resonance imaging detection of occult skin and subcutaneous abnormalities in juvenile dermatomyositis. Implications for diagnosis and therapy. Arthritis Rheum 2000;43(8):1866-73.

197. Summers RM, Brune AM, Choyke PL, Chow CK, Patronas NJ, Miller FW, et al. Juvenile idiopathic inflammatory myopathy: exercise-induced changes in muscle at short inversion time inversion-recovery MR imaging. Radiology 1998;209(1):191-6.

198. Pachman LM, Jonasson O, Cannon RA, Friedman JM. HLA-B8 in juvenile dermatomyositis. Lancet 1977;2(8037):567-8.

199. Reed AM, Pachman L, Ober C. Molecular genetic studies of major histocompatibility complex genes in children with juvenile dermatomyositis: increased risk associated with HLA-DQA1 *0501. Hum Immunol 1991;32(4):235-40.

200. Mamyrova G, O'Hanlon TP, Monroe JB, Carrick DM, Malley JD, Adams S, et al. Immunogenetic risk and protective factors for juvenile dermatomyositis in Caucasians. Arthritis Rheum 2006;54(12):3979-87.

201. Pachman LM, Liotta-Davis MR, Hong DK, Kinsella TR, Mendez EP, Kinder JM, et al. TNFalpha-308A allele in juvenile dermatomyositis: association with increased production of tumor necrosis factor alpha, disease duration, and pathologic calcifications. Arthritis Rheum 2000;43(10):2368-77.

202. Mamyrova G, O'Hanlon TP, Sillers L, Malley K, James-Newton L, Parks CG, et al. Cytokine gene polymorphisms as risk and severity factors for juvenile dermatomyositis. Arthritis Rheum. 2008;58(12):3941-3950.

203. Pachman LM, Hayford JR, Hochberg MC, Pallansch MA, Chung A, Daugherty CD, et al. New-onset juvenile dermatomyositis: comparisons with a healthy cohort and children with juvenile rheumatoid arthritis. Arthritis Rheum 1997;40(8):1526-33.

204. Mamyrova G, Rider LG, Haagenson L, Wong S, Brown KE. Parvovirus B19 and onset of juvenile dermatomyositis. JAMA 2005;294(17):2170-1.

205. Koch MJ, Brody JA, Gillespie MM. Childhood polymyositis: a case-control study. Am J Epidemiol 1976;104(6):627-31.

206. Bowles NE, Dubowitz V, Sewry CA, Archard LC. Dermatomyositis, polymyositis, and Coxsackie-B-virus infection. Lancet 1987;1(8540):1004-7.

207. Sallum AM, Kiss MH, Silva CA, Wakamatsu A, Sachetti S, Lotufo S, et al. MHC class I and II expression in juvenile dermatomyositis skeletal muscle. Clin Exp Rheumatol 2009;27(3):519-26.

208. Sallum AM, Kiss MH, Silva CA, Wakamatsu A, Vianna MA, Sachetti S, et al. Difference in adhesion molecule expression (ICAM-1 and VCAM-1) in juvenile and adult dermatomyositis, polymyositis and inclusion body myositis. Autoimmun Rev 2006;5(2):93-100.

209. Sallum AM, Marie SK, Wakamatsu A, Sachetti S, Vianna MA, Silva CA, et al. Immunohistochemical analysis of adhesion molecule expression on muscle biopsy specimens from patients with juvenile dermatomyositis. J Rheumatol 2004;31(4):801-7.

210. O'Connor KA, Abbott KA, Sabin B, Kuroda M, Pachman LM. MxA gene expression in juvenile dermatomyositis peripheral blood mononuclear cells: association with muscle involvement. Clin Immunol 2006;120(3):319-25.

211. Wedderburn LR, Varsani H, Li CK, Newton KR, Amato AA, Banwell B, et al. International consensus on a proposed score system for muscle biopsy evaluation in patients with juvenile dermatomyositis: a tool for potential use in clinical trials. Arthritis Rheum. 2007;57(7):1192-1201.

212. Tezak Z, Hoffman EP, Lutz JL, Fedczyna TO, Stephan D, Bremer EG, et al. Gene expression profiling in DQA1*0501+ children with untreated dermatomyositis: a novel model of pathogenesis. J Immunol 2002;168(8):4154-63.

213. Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. Mol Med 2007;13(1-2):59-68.

214. Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R, et al. Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. Ann Neurol 2005;57(5):664-78.

215. Walsh RJ, Kong SW, Yao Y, Jallal B, Kiener PA, Pinkus JL, et al. Type I interferon-inducible gene expression in blood is present and reflects disease activity in dermatomyositis and polymyositis. Arthritis Rheum 2007;56(11):3784-92.

216. Artlett CM, Ramos R, Jiminez SA, Patterson K, Miller FW, Rider LG. Chimeric cells of maternal origin in juvenile idiopathic inflammatory myopathies. Childhood Myositis Heterogeneity Collaborative Group. Lancet 2000;356(9248):2155-6.

217. Reed AM, McNallan K, Wettstein P, Vehe R, Ober C. Does HLA-dependent chimerism underlie the pathogenesis of juvenile dermatomyositis? J Immunol 2004;172(8):5041-6.

218. Reed AM, Picornell YJ, Harwood A, Kredich DW. Chimerism in children with juvenile dermatomyositis. Lancet 2000;356(9248):2156-7.

219. Rouster-Stevens KA, Pachman LM. Autoantibody to signal recognition particle in African American girls with juvenile polymyositis. J.Rheumatol. 2008;35(5):927-929.

220. O'Hanlon TP, Rider LG, Schiffenbauer A, Targoff IN, Malley K, Pandey JP, et al. Immunoglobulin gene polymorphisms are susceptibility factors in clinical and

autoantibody subgroups of the idiopathic inflammatory myopathies. Arthritis Rheum. 2008;58(10):3239-3246.

221. Rider LG. The heterogeneity of juvenile myositis. Autoimmun.Rev. 2007;6(4):241-247.

222. Oddis CV, Fertig N, Goel A, Espada G, Confalone Gregorian M, Maldonado Cocco MA. Clinical and serological characterisation of the anti-MJ antibody in childhood myositis (abstract). Arthritis Rheum 1997;40(9):S139.

223. Wedderburn LR, Rider LG. Juvenile dermatomyositis: new developments in pathogenesis, assessment and treatment. Best Pract Res Clin Rheumatol 2009;23(5):665-78.

224. Feldman BM, Rider LG, Reed AM, Pachman LM. Juvenile dermatomyositis and other idiopathic inflammatory myopathies of childhood. Lancet 2008;371(9631):2201-2212.

225. Espada G, Maldonado Cocco JA, Fertig N, Oddis CV. Clinical and serologic characterization of an argentine pediatric myositis cohort: identification of a novel autoantibody (anti-MJ) to a 142-kDa protein. J Rheumatol 2009;36(11):2547-51.

226. Scalapino K, Arkachaisri T, Lucas M, Fertig N, Helfrich DJ, Londino AV, Jr., et al. Childhood onset systemic sclerosis: classification, clinical and serologic features, and survival in comparison with adult onset disease. J Rheumatol 2006;33(5):1004-13.

227. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum 2005;35(1):35-42.

228. Targoff IN, Trieu EP, Levy-Neto M, Fertig N, Oddis CV. Sera with autoantibodies to the MJ antigen react with NXP2. Arthritis Rheum 2007;56(9):S787.

229. Fisler RE, Liang MG, Fuhlbrigge RC, Yalcindag A, Sundel RP. Aggressive management of juvenile dermatomyositis results in improved outcome and decreased incidence of calcinosis. J Am Acad Dermatol 2002;47(4):505-11.

230. Huemer C, Kitson H, Malleson PN, Sanderson S, Huemer M, Cabral DA, et al. Lipodystrophy in patients with juvenile dermatomyositis--evaluation of clinical and metabolic abnormalities. J Rheumatol 2001;28(3):610-5.

231. Singh S, Bansal A. Twelve years experience of juvenile dermatomyositis in North India. Rheumatol Int 2006;26(6):510-5.

232. Bingham A, Mamyrova G, Rother KI, Oral E, Cochran E, Premkumar A, et al. Predictors of acquired lipodystrophy in juvenile-onset dermatomyositis and a gradient of severity. Medicine (Baltimore) 2008;87(2):70-86.

233. Mamyrova G, Targoff IN, Lindsley CB, Wallace CA, Haftel H, Oral E, et al. Distinguishing clinical features of JDM associated with a myositis-associated autoantibody (MAA) directed against a 155 kd protein. Arthritis Rheum 2003;48(9):S1304.

234. Kimura Y, Sakai F, Nakano O, Kisaki O, Sugimoto H, Sawamura T, et al. The newly identified human nuclear protein NXP-2 possesses three distinct domains, the nuclear matrix-binding, RNA-binding, and coiled-coil domains. J.Biol.Chem. 2002;277(23):20611-20617.

235. Rosendorff A, Sakakibara S, Lu S, Kieff E, Xuan Y, DiBacco A, et al. NXP-2 association with SUMO-2 depends on lysines required for transcriptional repression. Proc.Natl.Acad.Sci.U.S.A 2006;103(14):5308-5313.

236. Fischer A, Meehan RT, Feghali-Bostwick CA, West SG, Brown KK. Unique characteristics of systemic sclerosis sine scleroderma-associated interstitial lung disease. Chest 2006;130(4):976-81.

237. Fischer A, Pfalzgraf FJ, Feghali-Bostwick CA, Wright TM, Curran-Everett D, West SG, et al. Anti-th/to-positivity in a cohort of patients with idiopathic pulmonary fibrosis. J Rheumatol 2006;33(8):1600-5.

238. Mitri GM, Lucas M, Fertig N, Steen VD, Medsger TA, Jr. A comparison between anti-Th/To- and anticentromere antibody-positive systemic sclerosis patients with limited cutaneous involvement. Arthritis Rheum 2003;48(1):203-9.

239. Poormoghim H, Lucas M, Fertig N, Medsger TA, Jr. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. Arthritis Rheum 2000;43(2):444-51.

240. Koenig M, Fritzler MJ, Targoff IN, Troyanov Y, Senecal JL. Heterogeneity of autoantibodies in 100 patients with autoimmune myositis: insights into clinical features and outcomes. Arthritis Res.Ther. 2007;9(4):R78.

241. Gunawardena H, Betteridge ZE, McHugh NJ. Myositis-specific autoantibodies: their clinical and pathogenic significance in disease expression. Rheumatology (Oxford) 2009;48(6):607-12.

242. Gunawardena H, Betteridge ZE, McHugh NJ. Newly identified autoantibodies: relationship to idiopathic inflammatory myopathy subsets and pathogenesis. Curr.Opin.Rheumatol. 2008;20(6):675-680.

243. Betteridge ZE, Gunawardena H, McHugh NJ. Pathogenic mechanisms of disease in myositis: autoantigens as clues. Curr Opin Rheumatol 2009;21(6):604-9.

244. Nakashima R, Imura Y, Kobayashi S, Yukawa N, Yoshifuji H, Nojima T, et al. The RIG-I-like receptor IFIH1/MDA5 is a dermatomyositis-specific autoantigen identified by the anti-CADM-140 antibody. Rheumatology (Oxford);49(3):433-40.

245. Wakasugi K, Slike BM, Hood J, Ewalt KL, Cheresh DA, Schimmel P. Induction of angiogenesis by a fragment of human tyrosyl-tRNA synthetase. J Biol Chem 2002;277(23):20124-6.

246. Zampieri S, Biral D, Adami N, Ghirardello A, Rampudda ME, Tonello M, et al. Expression of myositis specific autoantigens during post-natal myogenesis. Neurol.Res. 2008;30(2):145-148.

247. Burd CJ, Kinyamu HK, Miller FW, Archer TK. UV radiation regulates Mi-2 through protein translation and stability. J Biol Chem 2008;283(50):34976-82.

248. Casciola-Rosen LA, Pluta AF, Plotz PH, Cox AE, Morris S, Wigley FM, et al. The DNA mismatch repair enzyme PMS1 is a myositis-specific autoantigen. Arthritis Rheum. 2001;44(2):389-396.

249. Okada T, Noji S, Goto Y, Iwata T, Fujita T, Matsuzaki Y, et al. Immune responses to DNA mismatch repair enzymes hMSH2 and hPMS1 in patients with pancreatic cancer, dermatomyositis and polymyositis. Int J Cancer 2005;116(6):925-33.

250. Krajewska M, Wang HG, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, et al. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. Cancer Res 1997;57(8):1605-13.

251. Krajewski S, Gascoyne RD, Zapata JM, Krajewska M, Kitada S, Chhanabhai M, et al. Immunolocalization of the ICE/Ced-3-family protease, CPP32 (Caspase-3),

in non-Hodgkin's lymphomas, chronic lymphocytic leukemias, and reactive lymph nodes. Blood 1997;89(10):3817-25.

252. Mimura Y, Yazawa N, Tamaki Z, Ashida R, Jinnin M, Asano Y, et al. Anti-p53 antibodies in patients with dermatomyositis/polymyositis. Clin.Rheumatol. 2007;26(8):1328-1331.

253. Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 2009;136(1):123-35.

254. Park SG, Schimmel P, Kim S. Aminoacyl tRNA synthetases and their connections to disease. Proc Natl Acad Sci U S A 2008;105(32):11043-9.

255. Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 1976;17(1):62-70.

256. Rodova M, Ankilova V, Safro MG. Human phenylalanyl-tRNA synthetase: cloning, characterization of the deduced amino acid sequences in terms of the structural domains and coordinately regulated expression of the alpha and beta subunits in chronic myeloid leukemia cells. Biochem Biophys Res Commun 1999;255(3):765-73.

257. Miyaki M, Iijima T, Shiba K, Aki T, Kita Y, Yasuno M, et al. Alterations of repeated sequences in 5' upstream and coding regions in colorectal tumors from patients with hereditary nonpolyposis colorectal cancer and Turcot syndrome. Oncogene 2001;20(37):5215-8.

258. Wasenius VM, Hemmer S, Kettunen E, Knuutila S, Franssila K, Joensuu H. Hepatocyte growth factor receptor, matrix metalloproteinase-11, tissue inhibitor of

metalloproteinase-1, and fibronectin are up-regulated in papillary thyroid carcinoma: a cDNA and tissue microarray study. Clin Cancer Res 2003;9(1):68-75.

259. Scandurro AB, Weldon CW, Figueroa YG, Alam J, Beckman BS. Gene microarray analysis reveals a novel hypoxia signal transduction pathway in human hepatocellular carcinoma cells. Int J Oncol 2001;19(1):129-35.

260. Ray PS, Fox PL. A post-transcriptional pathway represses monocyte VEGF-A expression and angiogenic activity. EMBO J 2007;26(14):3360-72.

261. Takahashi K, Yoshida N, Murakami N, Kawata K, Ishizaki H, Tanaka-Okamoto M, et al. Dynamic regulation of p53 subnuclear localization and senescence by MORC3. Mol Biol Cell 2007;18(5):1701-9.

262. Clement JF, Meloche S, Servant MJ. The IKK-related kinases: from innate immunity to oncogenesis. Cell Res 2008;18(9):889-99.

263. Stone KB, Oddis CV, Fertig N, Katsumata Y, Lucas M, Vogt M, et al. Anti-Jo-1 antibody levels correlate with disease activity in idiopathic inflammatory myopathy. Arthritis Rheum 2007;56(9):3125-31.

264. Burd CJ, Kinyamu HK, Miller FW, Archer TK. UV radiation regulates Mi-2 through protein translation and stability. J.Biol.Chem. 2008.

265. Levine SM, Rosen A, Casciola-Rosen LA. Anti-aminoacyl tRNA synthetase immune responses: insights into the pathogenesis of the idiopathic inflammatory myopathies. Curr.Opin.Rheumatol. 2003;15(6):708-713.

266. Englund P, Wahlstrom J, Fathi M, Rasmussen E, Grunewald J, Tornling G, et al. Restricted T cell receptor BV gene usage in the lungs and muscles of patients with idiopathic inflammatory myopathies. Arthritis Rheum. 2007;56(1):372-383.

267. Krystufkova O, Vallerskog T, Helmers SB, Mann H, Putova I, Belacek J, et al. Increased serum levels of B cell activating factor (BAFF) in subsets of patients with idiopathic inflammatory myopathies. Ann Rheum Dis 2009;68(6):836-43.